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THE SEROTONIN TRANSPORTER KNOCKOUT RAT: MODELLING DEPRESSION FROM ETIOLOGY TO PHARMACOLOGICAL TREATMENT

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1. INTRODUCTION

1.1 Major depression.

Major depression is a severe psychiatric disorder that is the fourth leading cause of disability in the world, accounting for a relevant percentage of morbidity. It is a complex disorder characterized by the interaction of genetic, biological, social and environmental factors that play in concert to determine the development of the disease. Core symptoms include depressed mood, anhedonia (reduced ability to experience pleasure from natural rewards), irritability, difficulties in concentrating, abnormalities in appetite and sleep ('neurovegetative symptoms') and suicidal thoughts (Nestler, 2002). In addition to mortality associated with suicide, depressed patients are more likely to develop coronary artery disease and type 2 diabetes (Knol et al., 2006). The chronic, festering nature of depression contributes substantially to the global burden of disease and disability.

Despite the prevalence of depression and its considerable impact, the knowledge about its pathophysiology is poor compared with other chronic and potentially fatal multifactorial conditions. Available techniques to document the aberrant function of brain circuits depend on either post-mortem studies, which have numerous limitations, or neuroimaging techniques, which rely on detecting changes in neuronal activity by using indirect markers of activation (Phelps and LeDoux, 2005). Despite these difficulties, several brain regions and circuits regulating emotion, reward, executive function and dysfunctional changes within these highly interconnected 'limbic' regions have been implicated in depression and antidepressant action (Berton and Nestler, 2006). A large body of post-mortem (Sheline, 2003) and neuroimaging (Harrison, 2002) studies of depressed patients have reported reductions in grey-matter volume and glial density in the prefrontal cortex and in the hippocampus, regions thought to mediate the cognitive aspects of depression, such as feelings of worthlessness and guilt. Although these approaches have provided important insights into candidate brain regions, simple increases or decreases in regional brain activity

are probably insufficient to explain the complex array of symptoms caused by depression.

Therefore a clear pathogenetic mechanism is yet to be determined, but detailed studies have led to formulate different molecular theories of depression such as the "monoamine hypothesis" and the "neuroplasticity hypothesis".

The "monoamine hypothesis" of depression, which states that depression is caused by decreased monoamine function in the brain, originated from early clinical observations (Berton and Nestler, 2006; Pittenger and Duman, 2008). Two structurally unrelated compounds developed for non-psychiatric conditions, namely iproniazid and imipramine, had potent antidepressant effects in humans and were later shown to enhance central serotonin or noradrenaline transmission. Reserpine, an old antihypertensive agent that depletes monoamine stores, produced depressive symptoms in a subset of patients. Today's antidepressant agents offer a better therapeutic index and lower rates of side effects for most patients, but they are still designed to increase monoamine transmission acutely (Berton and Nestler, 2006), either by inhibiting neuronal reuptake (for example, selective serotonin reuptake inhibitors (SSRIs) such as fluoxetine) or by inhibiting degradation (for example, monoamine oxidase inhibitors such as tranylcypromine). However, while monoamine oxidase inhibitors and SSRIs produce immediate increases in monoamine transmission, their mood-enhancing properties require weeks of treatment. Moreover a relevant percentage of patients does not show an adequate response to antidepressant therapy, or relapses into the pathology, possibly due to persistent symptomatology. Although these monoamine-based agents are potent antidepressants, and alterations in central monoamine function might contribute marginally to genetic vulnerability (Lopez-Leon et al., 2007; Ruhe et al., 2007), the cause of depression is far from being a simple deficiency of central monoamines.

The "neuroplasticity hypothesis" of depression, suggests that mood disorders are caused by problems in information processing within particular neuronal circuits in the brain due to altered neuroplasticity, and that treatment with antidepressant drugs, may improve this deficit. Neuroplasticity is the ability of the brain to respond

and adapt to environmental challenges and encompasses a series of functional and structural mechanisms that may lead to neuronal remodeling, formation of novel synapses and birth of new neurons. Failure of such mechanisms might enhance the susceptibility to environmental challenges, such as stress, and ultimately lead to psychopathology. Neurotrophic factors play a key role as mediators of neuroplasticity. This aspect will be discussed in more detail later.

In recent years it has become more evident that many factors can contribute to the development of this disease, such as the association between a vulnerable genetic background and different environmental factors, such as stress.

Therefore, given the poor knowledge of the molecular mechanisms contributing to depression etiopathology and the unsuitability of the pharmacological treatment, several animal model of depression have been used, which could further our understanding of the pathophysiology of the disease, and also yield novel molecular mechanisms that may prove to be therapeutic targets for different antidepressants drugs.

1.2 Animal models of depression.

An ideal animal model of depression would have identical causative factors, symptomatology and treatment modalities of the pathology. A number of symptoms of this disease, however, are clearly not measurable in preclinical paradigms, such as recurrent thoughts of death or suicide, or excessive thoughts of guilt. It is clear that evolutionary progression has provided humans with a much more elaborated cerebral cortex that facilitates integration of complex psychological concepts also relevant to human depression, such as self-esteem and the ability to perceive the future, which are absent in rodents (Cryan and Holmes, 2005). Despite these difficulties, a number of diverse animal models of depression have been widely used, many of them showing substantial construct validity (i.e., antidepressant administration reverses the behavioural parameters assessed). These animal models are often referred to as tests of antidepressant-like activity, given that they all have been validated since the introduction of clinically approved medications (Cryan and Slattery, 2007). This also renders the predictive validity of the models unclear until a novel acting compound from preclinical testing is successfully applied in humans.

The most used animal models of depression are summarized in Table 1 (Calabrese et al., 2011).

Table 1. Traditional animal models used in depression research.

Animal model	Description
Forced swim test (FST)	Rodents are placed in an inescapable container of water. Swimming and climbing behaviours are evaluated and are increased with serotonergic or catecholaminergic antidepressants, respectively .
Tail suspension test (TST)	Rodents, chiefly mice, when hung from the tail will adopt an immobile posture. Antidepressant treatment increases the time animals spend in active behaviours.
Learned helplessness	Animals exposed to inescapable shocks subsequently fail to escape when able to. Antidepressant treatment increases the number of escapes, not all animals develop this helpless behavior.
Olfactory bulbectomy	Removal of the olfactory bulbs causes a constellation of behavioural and neurochemical alterations, which are only reversed by chronic antidepressant treatment
Social stress	Mice form stable subordinate relationship with a dominant, show behavioural and neurochemical alterations similar to depressed patients. A number of these alterations can be reversed with antidepressant treatment but the model requires further validation.
Social isolation stress	Animals were housed in their cages alone and are deprived of any social interaction. This model induce anxiety and depressive -like behaviors, that are reversed by antidepressant treatment.
Chronic mild stress	Animals are subjected to a variety of unpredictable stressors, which leads to a constellation of symptoms, that are reversed by antidepressant treatment.
Prenatal stress	Pregnant dams are usually exposed to different types of stressors from embryonic day 14 (E14) until delivery. Prenatally-stressed rats show a higher degree of immobility in the FST and reduced explorative behavior.
Early life stress (ELS)	When animals are separated from the mother during early postnatal life they can develop a number of depression-like behavioural characteristics. These behaviours are not present in all animals subjected to this treatment.

In the forced swim test (FST, also called the Porsolt test) animals are subjected to two trials during which they are forced to swim in an acrylic glass cylinder filled with water, and from which they cannot escape. The time that the animals spend without moving is associated to depressed behaviour. This immobility time is decreased by antidepressants.

The tail suspension test (TST) is used to assay mood levels in rodents. Changes in immobility time indicate changes in mood. It is widely used to detect potential antidepressant effects of drugs.

In the learned helplessness experiment an animal is repeatedly hurt by an adverse stimulus, which it cannot escape. Eventually the animal will stop trying to avoid the pain and behave as if it is utterly helpless to change the situation. Finally, when opportunities to escape are presented, this learned helplessness prevents any action. Chronic antidepressant treatment is able to revert this behavior in most of the cases (Nestler and Hyman, 2010).

Chronic mild or chronic unpredictable stress involves subjecting normal rodents to a series of repeated stresses (food or water deprivation, crowding, isolation, soiled caged, restraint, foot shock or cold temperature) over a period of weeks or longer that leads to a chronic depressive-like state developed gradually over time (Willner, 2005). At the end of the stress, the animals show signs of anhedonia (for example, reduced sucrose preference; face validity), which can be reversed by chronic, but not acute, administration of antidepressant medications (predictive validity).

Chronic social defeat stress involves subjecting rodents to repeated bouts of social subordination, after which the rodents show a range of depression-like symptoms, including anhedonia and social withdrawal, which can be reversed by chronic, but not acute, antidepressants (Krishnan et al., 2007). Chronic social defeat also induces a metabolic syndrome in mice characterized by weight gain and insulin and leptin resistance (Chuang et al., 2010), consistent with homeostatic abnormalities observed in depression. A further advantage of chronic social defeat is that it can be used to study 'resilience', as a subset of mice, subjected to the same stress, fail to develop behavioral and metabolic disturbances. In contrast with all of these forms of 'active'

stress, it was recently found that prolonged exposure (weeks to months) of adult rodents to social isolation induces anhedonia that can be treated effectively with chronic antidepressants (Wallace et al., 2009).

In the search for etiologically valid animal models of major depression, in recent years much hope has been placed in our ability to harness the power of genomics to resolve the genetic architecture of major depression heritability (estimated to 40%) and to identify causal mutations that can be reproduced in animals to emulate (in interaction with stress inducers discussed above) various aspect of behavioral and neural depression phenotypes. Several depression-like phenotypes have been reported in animals carrying mutations, which replicate naturally occurring single-nucleotide polymorphisms that alter the function of candidate genes, such as the Brain Derived Neurotrophic Factor (BDNF). In particular BDNF Val66Met knock-in mice represent a strong model of predisposition to depression since these animals displayed hypothalamic-pituitary-adrenal axis hyperreactivity, increased depressive-like and anxiety-like behaviors, (Soliman et al., 2010; Yu et al., 2012). In the context of mood disorder the serotonin transporter gene is also particularly relevant. The human serotonin transporter is encoded by a single gene, SLC6A4 (solute carrier family 6 member 4), whose transcriptional activity is modulated by genetic variants, including a functional polymorphism in the promoter region (5-HTTLPR), characterized by a long (L) and a short (S) variant. Specifically, functional studies confirmed that the L allele of the functional polymorphism 5-HTTLPR is associated with higher levels of transcriptional activity and higher rate of serotonin uptake with respect to the S variant (Murphy et al., 2008). Moreover, the L allele could be a protective factor for major depression, whereas the low-functioning allele is associated with increased disease susceptibility upon exposure to adverse life events (Caspi et al., 2003; Uher and McGuffin, 2008). Given that this polymorphism is present in humans and non-human primates, but not in rodents, rats and mice with a partial or total deletion of the SERT gene have been generated. Animal with a total deletion of the serotonin transporter displayed increased levels of anxiety- and depression-like behaviors (Holmes et al., 2003; Lira et al., 2003; Olivier et al., 2003).

For instance SERT$^{-/-}$ rats spent less time in the center part of the open field as well as on the open arm of the plus maze, suggesting an enhanced level of anxiety (Olivier et al., 2008).

Several procedures have also disrupted an animal's glucocorticoid homeostasis, based on derangements of the HPA axis in depression. To mimic the human situation of altered GR function claimed for depression, GR-heterozygous mutant mice (GR$^{+/-}$) have been generated. These GR$^{+/-}$ mice have a predisposition for depression (Ridder, et al., 2005; Chourbaji and Gass, 2007; Molteni et al., 2010b), supporting the hypothesis that a dysfunction of corticosteroid receptors is involved in the pathogenesis of stress-related psychiatric disorders (Holsboer, 2000; DeRijk and de Kloet, 2008). Interestingly, behavioural abnormalities in these animals are not found at baseline, but manifest only after stress exposure, in agreement with the idea that gene * environment interaction is critical for the aetiology of mood disorders (Caspi, et al., 2003).

Other animal models of depression are based on the evidence that stressful experiences during gestation or early in life can lead to enhanced susceptibility for mental illness. The negative influence exerted by adverse life events during gestation on brain development has received a great deal of attention since development represents a critical moment for shaping adult behavior and may set the stage to disease vulnerability later in life. In the majority of prenatal stress studies, pregnant dams have been exposed to stress as adverse manipulation and the short and long-term behavioral and molecular changes in the newborns have been examined. Dams are usually exposed to different types of stressors (repeated daily immobilization under bright light, exposure to noise, sleep deprivation, immersion in cold-water) during the last week of gestation, from embryonic day 14 (E14) until delivery, although shorter periods of stress exposure have also been employed. Morley-Fletcher and coworkers observed that prenatally-stressed rats show a higher degree of immobility in the forced swim test, if compared to rats with normal gestation (Morley-Fletcher et al., 2003). Similarly, prenatal stress reduces explorative behavior in adult rats, presumably due to decreased motivation (Patin et al., 2004; Secoli and

Teixeira, 1998; Vallee et al., 1997), a symptom often present in depressed patients. Moreover short- and long-term effects produced by prenatal stress may be the consequence of a direct interference with neurodevelopmental processes, but could also result from changes in post-partum maternal care (Champagne and Meaney, 2006).

The effects of early life stress (ELS) at adulthood have been investigated through a variety of experimental manipulations, the vast majority being based on the interference in mother-pup interaction, the so-called maternal deprivation (MD) paradigm. This procedure involves the removal of the pups from their mothers between birth and weaning for different periods of time, followed by testing the behavioral and molecular consequences when reaching adulthood. The best-studied paradigm consists of periods of daily separation (usually 3 hours) performed from postnatal day (PND) 2 to postnatal day 14, although a single 24 h period of removal at different postnatal ages has also been frequently employed. MacQueen and collaborators have shown that mice exposed to maternal separation for 3 h/day from PND 4 to PND 21 display, as adults, shorter swim time in the forced swim test, a behavioral despair that resembles depressive-like behavior (MacQueen et al., 2003). Moreover early postnatal stress led to changes in fear and emotional reactivity, which could also interfere with the cognitive test (Kosten et al., 2006; Romeo et al., 2003).

1.3 Neuronal plasticity and BDNF.

Neuronal plasticity refers to the ability of the nervous system to respond and adapt to environmental challenges and encompasses a series of functional and structural mechanisms that may lead to neuronal remodeling, formation of novel synapses and birth of new neurons. However, in a broader sense, neuronal plasticity is intimately linked to cellular responsiveness and may therefore be considered an index of the neuronal capability to adapt its function to a different demand. Failure of such mechanisms might enhance the susceptibility to environmental challenges, such as stress, and ultimately lead to psychopathology. That is to say that the brain, or more specifically, some brain structures or circuits, may become more vulnerable by loosing progressively (or suddenly) the ability to adapt and maintain their homeostasis. The manifestation of such vulnerability can be quite different according to the pathologic condition and may lead to overt degeneration (as occurring in neurodegenerative disorders) or to more subtle changes, leading to functional impairment, which represents a feature of psychiatric illness.

Although the individual susceptibility threshold to environmental events may be genetically determined, it is believed that life events occurring during brain development may be critical for later psychopathology. Traumatic experiences occurring early in life may herein disrupt the correct program of maturation and eventually impact on brain function leading to a deterioration of neuronal plasticity. Indeed the brains of depressed subjects show structural abnormalities and reduced expression of several markers for neuronal function and viability, among which neurotrophic factors seem to be playing a pivotal role (aan het Rot et al., 2009; Sheline et al., 2003).

Neurotrophic factors (NTFs), and in particular the neurotrophin family, are activity-regulated genes that play an important role in network construction and reorganization, which are the main processes involved in neuroplasticity. In fact, besides their classical role in supporting neuronal survival, NTFs finely modulate all the crucial steps of network construction, from neuronal migration to experience-

dependent refinement of local connections (Poo, 2001). These functions were first reported based on the observation that during the development of the nervous system, neuron survival depends on the limited amount of specific NTFs secreted by target cells (Huang and Reichardt, 2001). However, it is now well established that NTFs are important mediators of neuronal plasticity also in adulthood where they modulate axonal and dendritic growth and remodeling, membrane receptor trafficking, neurotransmitter release, synapse formation and function (Lu et al., 2005). The neurotrophin BDNF has emerged as crucial mediator of neuronal plasticity, not only because it is abundant in brain regions that are particularly relevant for plasticity, but because it shows a remarkable activity-dependent regulation of its expression and secretion (Bramham and Messaoudi, 2005), suggesting that it might indeed bridge experience with enduring change in neuronal function.

BDNF has a sophisticated organization in terms of transcriptional, translational and post translational regulatory mechanisms. With regard to BDNF transcription, its gene consists of nine 5' untranslated exons, each linked to individual promoter regions, a 3' coding exon (IX), which codes for the BDNF pre-protein amino acid sequence, and two polyadenylation sites at the 3' UTR (Aid et al., 2007). The transcription of each exons is driven by separate promoters in turn controlled by an array of signaling mechanisms, including Ca^{2+}, CREB, CaRF, Npas4, USF, MeCP2, CaMKII and hormones (Zhou et al., 2006; Molteni et al., 2010a; Pruunsild et al., 2011). Furthermore it has been demonstrated that the transcription of specific BDNF splice variants is controlled by a variety of epigenetic mechanisms, including DNA methylation and post translational modifications of histones (Lubin et al., 2008; Molteni et al., 2010a; Roth et al., 2009). The regulation of specific promoters causes the temporal and spatial expression of specific BDNF transcripts (Lauterborn et al., 1996), some of which can undergo trafficking and targeting to dendrites (Chiaruttini et al., 2008), a process that may also be influenced by the 3' untranslated region (UTR) of the neurotrophin (Ghosh et al., 1994; An et al., 2008).
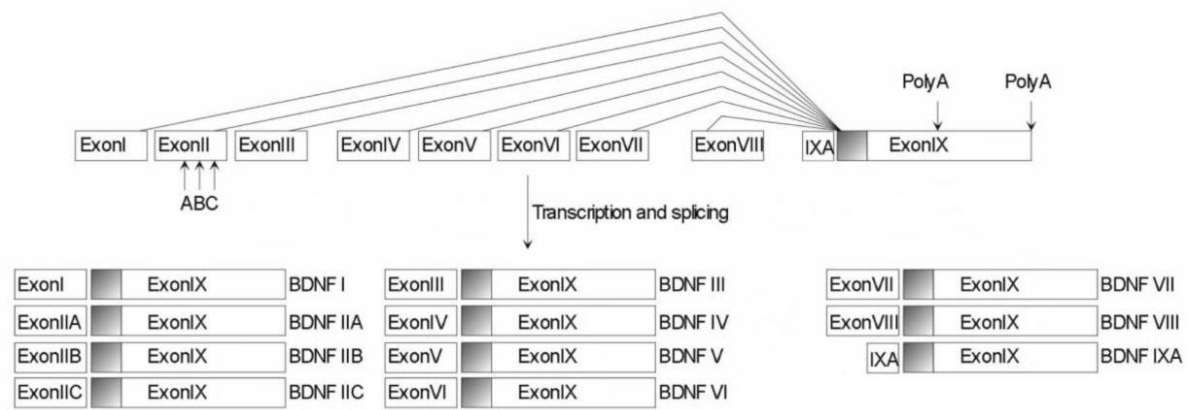
Fig.1 Gene structure of BDNF and its transcripts.

The BDNF protein is initially synthesized as proform that can either be cleaved into the mature neurotrophin or transported to the plasma membrane and released in an unprocessed manner. Several matrix metalloproteases (MMP) as well as plasmin are responsible for extracellular cleavage of the neurotrophin, whereas furin and specific proconvertases control its intracellular processing (Schweigreiter, 2006). Differently from other neurotrophins, BDNF can be secreted through a constitutive as well as a regulated pathway. The prodomain interacts with sortilin that promote an appropriate configuration of proBDNF. This, in turn, allows the sorting motif in the mature domain of BDNF to interact with carboxypeptidase E (CPE), and, therefore, to sort BDNF into the regulated secretory pathway. Thus, BDNF protein can be packaged into secretory vescicles (Lessmann et al., 2003) that are present in both axon terminals (presynaptic site) and dendrites (postsynaptic site) of glutamatergic neurons (Fawcett et al., 1997), from which the neurotrophin can be released in a Ca^{2+}-dependent manner. Upon release, proBDNF and mBDNF have divergent activities, since the former binds with high affinity to p75NTR leading to apoptosis, whereas mBDNF binds to TrkB receptors promoting cell survival (Lu et al., 2005). Activated receptors in general are capable of triggering a number of signal transduction cascades including the mitogen-activated protein kinase (MAPK) pathway, the phosphatidylinositol 3-kinase (PI3K) pathway and the phospholipase C-γ (PLC-γ) pathway (Huang and Reichardt, 2001; Huang and Reichardt, 2003).
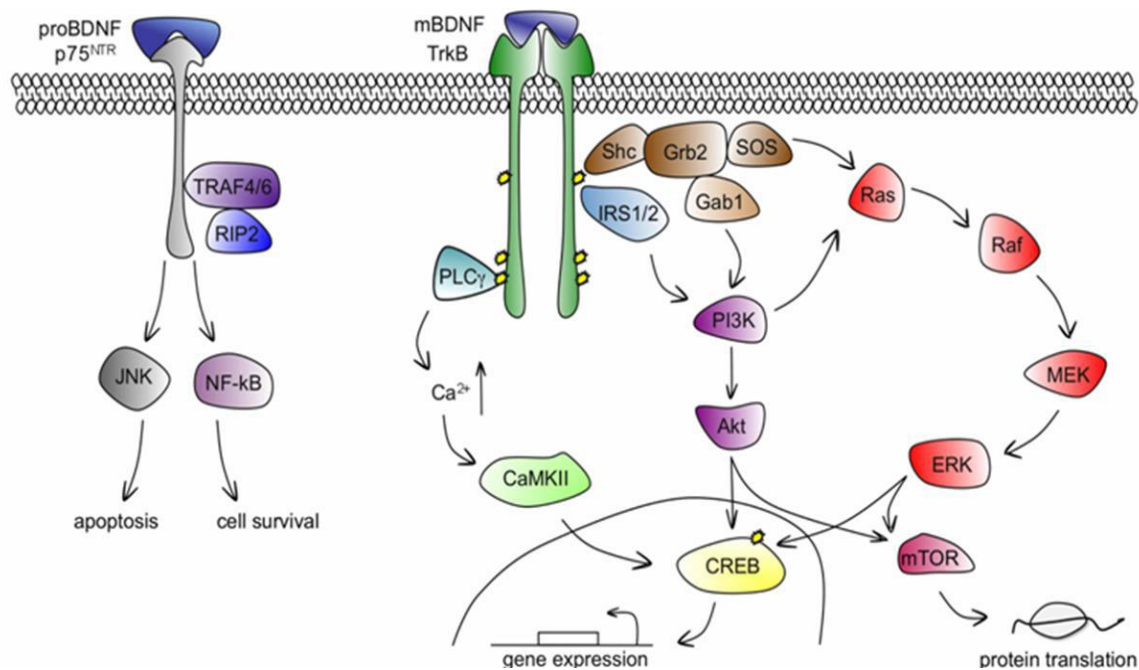
Fig.2 Intracellular signaling pathways activated by the binding of proBDNF with p75NTR and mBDNF with TrkB.

An impairment of hippocampal BDNF signaling produces depression-related behaviors and impairs the actions of antidepressants (Monteggia et al., 2007; Taliaz et al., 2010). Hippocampal BDNF levels are reduced in postmortem samples from depressed humans (Karege et al., 2005). On the other hand experimental increases in hippocampal BDNF levels produce antidepressant-like effects (Hoshaw et al., 2005). BDNF's role in depression has also been extensively studied in mutant mice. Lack of BDNF is not sufficient to produce a depressive phenotype (Chourbaji et al., 2004), but the neurotrophin is required for the behavioural response to antidepressant since the selective deletion of the BDNF gene in the hippocampal dentate gyrus, as well as the impaired function of its high affinity receptor TrkB, prevents behavioural responses to antidepressant drugs (Adachi et al., 2008; Monteggia et al., 2004; et al., 2003).

2. AIM OF THE STUDY

Major depression is a complex disorder characterized by the interaction of genetic, biological, social and environmental factors, which play in concert to determine the development of the disease. The serotonin transporter gene is of particular relevance in the context of mood disorders because it is the target of several antidepressant drugs and, due to the presence of a functional polymorphism within its promoter region, it has been associated with different neuropsychiatric disorders (Caspi et al., 2003; Uher and McGuffin, 2008). Since the molecular mechanisms contributing to depression pathophysiology are still poorly understood, several genetic or environmental animal models of depression have been developed in order to reproduce the depressive phenotype and to characterize the molecular alterations of the disease. Among these, rodents (mice or rats) with a total deletion of the serotonin transporter represent a good model of anxiety and depression phenotypes and they have been extensively characterized at behavioral level (Holmes et al., 2003; Lira et al., 2003; Olivier et al., 2008), although a detailed analyses of the molecular phenotype associated with genetic alterations of SERT is still lacking. In particular, limited information exists on potential changes in neuronal plasticity, which is strongly associated with depression. Indeed it has been proposed that psychopathology may be associated with reduced expression and function of key mediators of neuronal plasticity, such as the neurotrophin brain-derived neurotrophic factor (BDNF), (McClung and Nestler, 2008; Pittenger and Duman, 2008).

Given all these premises, in this study, we investigated the expression of BDNF in different brain regions of adult SERT mutant rats (+/− and −/−). Moreover, since the neurotrophin has a complex genomic structure (Aid et al., 2007), we have investigated the influence of SERT deletion on its different transcripts and evaluated the involvement of several transcription factors in BDNF expression changes found in SERT mutant animals.

Moreover, considering that the depressive phenotype of SERT mutant animals appears to originate from the lack of the transporter during early life (Ansorge et al., 2004; Ansorge et al., 2008), we also assessed when the neuroplastic alteration of SERT$^{-/-}$ animals are established, by investigating the expression of BDNF in SERT knockout rats during postnatal development.

One system lying downstream from Npas4 and BDNF that may be relevant for the phenotype of SERT mutants is GABA. Indeed, Npas4 regulates the development of GABAergic synapses (Lin et al., 2008), while BDNF alteration can affect GABAergic cellular architecture and transmission (Sakata et al., 2009). Therefore we also established if SERT mutant rats are characterized by alterations in the expression of key GABAergic markers that may eventually contribute to the depressive phenotype of these animals.

A further aspect of the experimental work is aimed at addressing the issue of gene * environment interaction in mood disorders. Indeed, stressful experiences can exacerbate or precipitate depressive disorder in genetically-prone individuals (Caspi et al., 2003). In particular adverse events early in life may impact on brain structures that are not completely matured resulting critical for the development of psychopathology later in life. In order to establish if the molecular phenotype associated with deletion of SERT gene could be modulated by early life events, we exposed SERT mutant rats (+/− and −/−) to maternal deprivation, a strong form of stress, and we assessed the expression of the neurotrophin BDNF as marker of neuronal plasticity.

Last we investigated if pharmacological intervention in SERT mutant rats may restore neuroplastic defects associated with the vulnerable genotype. Therefore we chronically treated SERT knockout rats with the antidepressant duloxetine (a SNRI, dual blocker of the reuptake of the serotonin and norepinephrine) and we assessed its ability to normalize the expression of different neuroplastic genes that are significantly altered in mutant rats.

3. MATERIALS AND METHODS

3.1 Serotonin transporter-knockout rats.

SERT-knockout rats (Slc6a41Hubr) were generated by ENU-induced mutagenesis (Smits et al, 2006). All animals 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 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 06:00 h).

For basal analysis $SERT^{+/-}$, $SERT^{-/-}$ rats, and their wild-type controls ($SERT^{+/+}$), were sacrificed between 10 and 11 a.m. at adulthood (PND 90) whereas another cohort of $SERT^{+/+}$ and $SERT^{-/-}$ rats was sacrificed at postnatal days (PND) 0, 7, 14, 21, 28, at the same timeslot.

Brain regions of interest (ventral hippocampus, dorsal hippocampus and prefrontal cortex) were rapidly dissected. Dorsal hippocampus corresponds to plates 26-33 (from Bregma -2.12 to Bregma -3.80), while ventral hippocampus corresponds to plates 34-43 (from Bregma -4.16 to Bregma -6.30) according to the atlas of Paxinos and Watson (1996). Prefrontal cortex (defined as Cg1, Cg3, and IL sub-regions corresponding to the plates 6-10 according to the atlas of Paxinos and Watson (1996)) was dissected from 2-mm thick slices. The brain specimens were frozen on dry ice and stored at -80°C for 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).

3.2 Maternal deprivation stress paradigm.

The SERT$^{+/+}$, SERT$^{+/-}$ and SERT$^{-/-}$ animals were bred and reared in the Central Animal Laboratory of the Radboud University Nijmegen Medical Centre in Nijmegen, The Netherlands. From postnatal day 2 to postnatal day 14 rats were separated from their mothers for 3 hours a day, while rats from the control group were briefly handled during bedding change. After this manipulation, all the animals underwent normal animal care and were sacrificed at adulthood (PND 90). Brain regions of interest (ventromedial prefrontal cortex and dorsomedial prefrontal cortex) were rapidly dissected. Ventromedial prefrontal cortex (IL cortex) and dorsomedial prefrontal cortex (PrL cortex) correspond to plates 9-11 (from Bregma -3.24 to Bregma -3.92) according to the atlas of Paxinos and Watson (1996)). The brain specimens were frozen on dry ice and stored at -80°C for further analysis.

3.3 Pharmacological treatment of Serotonin transporter-knockout rats.

Fluoxetine administration at adulthood in SERT$^{+/+}$ and SERT$^{-/-}$ rats.
To mimic the effect of SERT blockade at adulthood, separate cohorts of adult wild type and SERT$^{-/-}$ rats were randomly assigned to receive daily injections (between 9:00 and 10:00 a.m.) of the selective-serotonin reuptake inhibitor fluoxetine (10 mg/kg i.p.) or saline for 3 weeks. Twenty-four hours after the last injection the animals were sacrificed and the prefrontal cortex was 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. The brain specimens were frozen on dry ice and stored at -80°C for further analysis.

Duloxetine treatment in 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). Twenty-four hours after the last injection the animals were sacrificed and the brain regions of interest (whole hippocampus and 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°C for further analysis.

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

Total RNA was isolated by single step of guanidinium isothiocyanate/phenol extraction using PureZol RNA isolation reagent (Bio-Rad Laboratories s.r.l. Italia) according with the manufacturer's instructions and quantified by spectrophotometric analysis. Following total RNA extraction, the samples were processed for real-time polymerase chain reaction (PCR) to assess total Brain derived neurotrophic factor (BDNF), long 3' UTR BDNF, BDNF exons I, IV, VI, neuronal PAS domain protein 4 (Npas4), cAMP responsive element-binding protein (CREB), calcium-responsive transcription factor (CaRF), γ2 subunit of GABA$_A$ receptor (GABA$_A$ γ2), Glutamic acid decarboxylase 1 (Gad67), Vescicular-GABA Transporter (Vgat), Parvalbumin (Pvalb) and 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, Italy) using the iScriptTM one-step RT-PCR kit for probes (Bio-Rad Laboratories). Samples were run in 384 well formats in triplicate as multiplexed reactions with a normalizing internal control (36B4 or 18S). Probe and primer sequences used (Table 1) were purchased from Eurofins MWG-Operon (Germany) and 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 (Ct) method was used to calculate the relative target gene expression.

3.5 Preparation of protein extract and Western blot analysis.

Western blot analysis was used to investigate BDNF protein levels in the total homogenate and in the crude synaptosomal fraction. Tissues were manually homogenized using a glass-glass potter in a pH 7.4 cold buffer containing 0.32 M sucrose, 0.1 mM EGTA, 1mM HEPES solution in presence of a complete set of protease (Roche) and phosphatase (Sigma-Aldrich) inhibitors. The total homogenate was centrifuged at 2,500 rpm for 10 min at 4°C. The supernatant obtained was centrifuged at 10,000 g for 15 min at 4°C to obtain a pellet (P2) corresponding to the crude synaptosomal fraction which was re-suspended in a buffer (20mM HEPES, 0.1 mM dithiothreitol (DTT), 0.1 mM EGTA) with protease and phosphatase inhibitors. Total protein content was measured according to the Bradford Protein Assay procedure (Bio-Rad Laboratories), using bovine serum albumin as calibration standard.

Equal amounts of protein were run under reducing conditions on the criterion TGX precast gels (Bio-Rad Laboratories) and then electrophoretically transferred onto PVDF membranes (Bio-Rad Laboratories). The blots were blocked with 10% nonfat dry milk and then incubated with the anti-BDNF rabbit polyclonal antibody (1:500, 4°C, overnight; Santa Cruz Biotechnology) able to recognize both the mature form of the neurotrophin (mBDNF; 14 kDa) and its precursor (proBDNF; 32 kDa). Membranes were then incubated for 1 h at room temperature with a peroxidase-conjugated anti-rabbit IgG (1:1.000) and immunocomplexes were visualized by chemiluminescence using the Chemidoc MP Imaging System (Bio-Rad Laboratories). Results were standardized using β-actin as the control protein, which was detected by evaluating

the band density at 43 kDa after probing the membranes with a mouse polyclonal antibody (1:10.000, Sigma–Aldrich) followed by a 1:10.000 dilution of peroxidase-conjugated anti-mouse IgG (Sigma–Aldrich). Protein levels were calculated by measuring the optical density of the autoradiographic bands using Quantity One software (Bio-Rad Laboratories). To ensure that autoradiographic bands were in the linear range of intensity, different exposure times were used.

3.6 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), one-way analysis of variance (ANOVA) followed by Bonferroni Post Hoc Test or Student t-test. Pearson product moment correlations (r) between levels of Npas4 mRNAs and $GABA_A$-$\gamma 2$ mRNAs were performed to evaluate the correlation between the expression levels of the two genes in single animals. Significance for all tests was assumed for $p<0.05$ Data are presented as means $\pm$ standard error (S.E.M.).

Table 2. Sequences of forward and reverse primers and probes used in Real-Time PCR analysis.

a: forward and reverse primers and probes purchased from Eurofins MWG-Operon			
Gene	**Forward primer**	**Reverse Primer**	**Probe**
Total Bdnf	5'-AAGTCTGCATTACATTCCTCGA-3'	5'-GTTTTCTGAAAGAGGGACAGTTTAT-3'	5'-TGTGGTTTGTTGCCGTTGCCAAG-3'
Npas4	5'-GGAAGTTGCTATACCTGTCGG-3'	5'-GTCGTAAATACTGTCACCCTGG-3'	5'-CATAGAATGGCCCAGATGCTCGCT-3'
Creb	5'-AGATTCTAGTGCCCAGCAAC-3'	5'-CTGTGCGAATCTGGTATGTTTG-3'	5'-TGTTCAAGCTGCCTCTGGTGATGT-3'
Carf	5'-GAGATGCACACACCATTCCA-3'	5'-GTGTTGGCTCATTGGGTTCT-3'	5'-CAGCCATCCAGCTCTTGTTGAAGA-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'
18S	5'-GTAACCCGTTGAACCCCATT-3'	5'-CCATCCAATCGGTAGTAGCG-3'	5'-TGCAATTATTCCCCATGAACGAGG-3'
36B4	5'-TTCCCACTGGCTGAAAAGGT-3'	5'-CGCAGCCGCAAATGC-3'	5'-AAGGCCTTCCTGGCCGATCCATC-3'
b: forward and reverse primers and probes purchased from Life Technologies			
Gene	**Accession number**		**Assay ID**
Long 3'UTR Bdnf	EF125675		Rn02531967_s1
Bdnf exon I	EF125675		Rn01484924_m1
Bdnf exon IV	EF125678		Rn01484927_m1
Bdnf exon VI	EF125680		Rn01484928_m1

4. RESULTS

4.1 Lack of serotonin transporter is associated with reduced basal Brain Derived Neurotrophic Factor (BDNF) expression in different brain regions.

Animals with SERT gene deletion represent an important tool to identify the molecular mechanisms contributing to the association between mood disorders and the life-long dysfunction of the serotonin transporter. Indeed SERT knockout rodents show depression- and anxiety-related behaviour (Olivier et al., 2008, Kalueff et al., 2009) supporting the possibility that altered function of SERT may indeed be associated with increased risk for mood disorders (Gardier, 2009).

We initially investigated possible changes in the expression of the neurotrophin BDNF, a key marker of neuroplasticity. Indeed, during the last few years it has become apparent that several psychiatric conditions, such as mood disorders, are associated with deficits or impairment of neuronal plasticity (Krishnan and Nestler, 2008; Pittenger and Duman, 2008, Calabrese et al., 2009). Our analyses were performed in the ventral and the dorsal hippocampus, two different hippocampal sub regions with different anatomical connectivity and functional implications (Fanselow and Dong 2010), and in the prefrontal cortex of SERT$^{+/-}$ and SERT$^{-/-}$ animals. Total BDNF mRNA levels are significantly reduced in the ventral hippocampus of SERT$^{-/-}$ rats (−50% vs SERT$^{+/+}$, $p<0.05$) (Fig.3A) but not in the dorsal hippocampus (−9% vs SERT$^{+/+}$, $p>0.05$) (Fig.3B). The neurotrophin is also significantly reduced in the prefrontal cortex of adult SERT$^{+/-}$ (-16% vs SERT$^{+/+}$, $p<0.001$) and SERT$^{-/-}$ rats (-34% vs SERT$^{+/+}$, $p<0.001$) (Fig.3C).

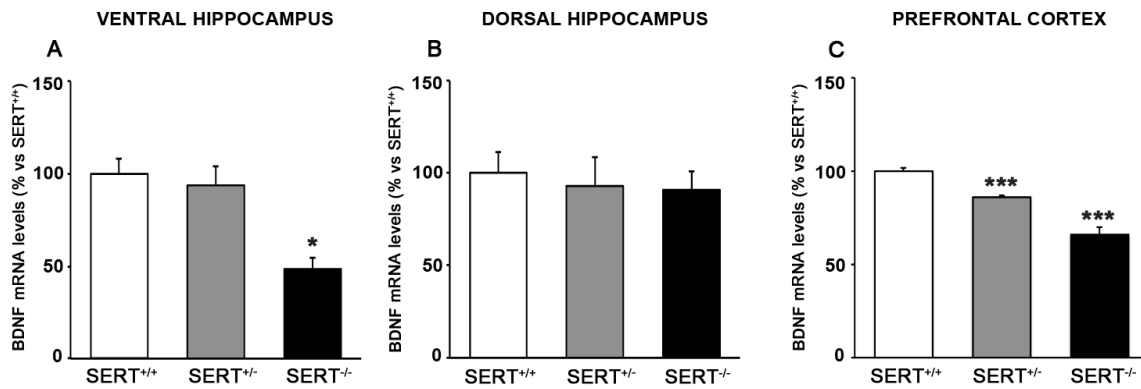**VENTRAL HIPPOCAMPUS**   **DORSAL HIPPOCAMPUS**   **PREFRONTAL CORTEX**

Fig.3 Deletion of SERT gene modulates BDNF gene expression in ventral hippocampus, dorsal hippocampus and in the prefrontal cortex.

Total BDNF mRNA levels were measured in the ventral hippocampus (A), in the dorsal hippocampus (B) and in the prefrontal cortex (C) of SERT$^{+/-}$ and SERT$^{-/-}$ rats, as compared to their wild-type (SERT$^{+/+}$) counterparts. The data, expressed as a percentage of +/+ animals (set at 100%), are the mean±SEM from 6 to 14 independent determinations. $*p<0.05$, $***p<0.001$ vs SERT$^{+/+}$ rats (one-way ANOVA with Bonferroni post hoc test).

It is known that the BDNF gene has a complex organization, and it is transcribed from at least eight promoters, which drives the transcription of a 5' exon alternatively spliced onto a common 3' exon-encoding BDNF protein (Aid et al., 2007). On this basis, we investigated the expression levels of the long 3' UTR BDNF and of the exons I, IV and VI since, up to date, their promoters have been well characterized (Takeuchi et al., 2002, Pruunsild et al., 2011).

In the ventral hippocampus the mRNA levels of exon I and exon IV are significantly reduced in SERT$^{-/-}$ rats (respectively -37% vs SERT$^{+/+}$, $p<0.05$; -35% vs SERT$^{+/+}$, $p<0.01$) (Fig.4B,C), while long 3' UTR BDNF and exon VI are not modulated in mutant rats (respectively +2% vs SERT$^{+/+}$, $p>0.05$; -14% vs SERT$^{+/+}$, $p>0.05$) (Fig.4A,D). Moreover exon I expression is also reduced in SERT heterozygous rats (-34% vs SERT$^{+/+}$, $p<0.05$).
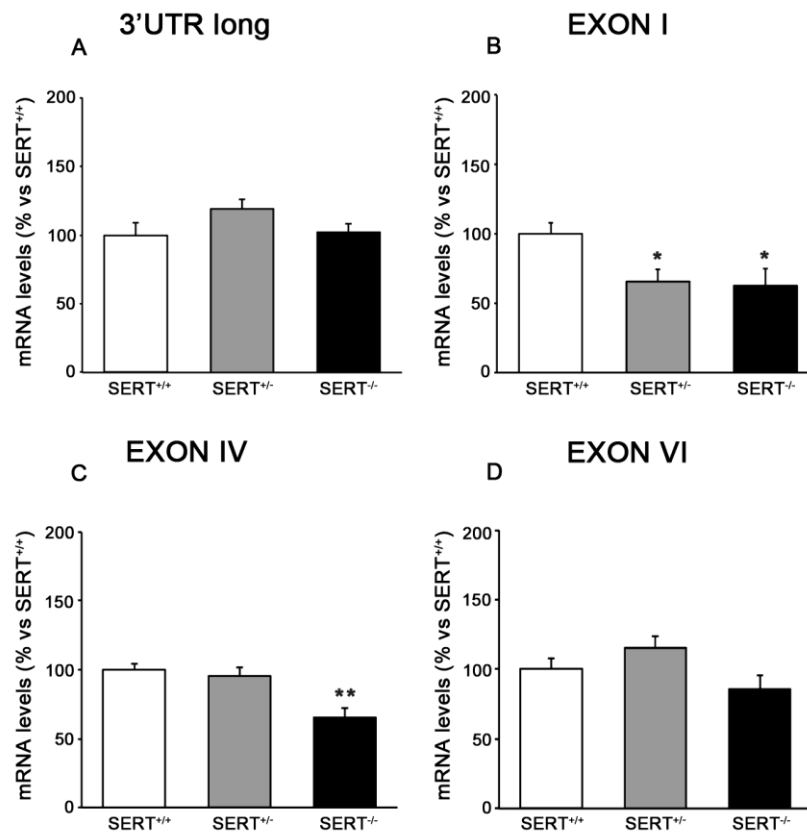
Fig.4 Deletion of SERT gene modulates long 3'UTR BDNF, exon I, exon IV and exon VI gene expression in the ventral hippocampus.

Long 3'UTR BDNF (A), exon I (B), exon IV (C) and exon VI (D) mRNA levels were measured in the ventral hippocampus of SERT$^{+/-}$ and SERT$^{-/-}$ rats, as compared to their wild-type (SERT$^{+/+}$) counterparts. The data, expressed as a percentage of +/+ animals (set at 100%), are the mean±SEM from 6 to 8 independent determinations. *p<0.05, **p<0.01 vs SERT$^{+/+}$ rats (one-way ANOVA with Bonferroni post hoc test).

In the dorsal hippocampus we found a significant reduction of BDNF exon VI in SERT$^{+/-}$ rats (-40% vs SERT$^{+/+}$, p<0.05), but not in SERT$^{-/-}$ (-33% vs SERT$^{+/+}$, p>0.05) (Fig.5D). On the other hand the other three transcripts are not influenced by the lack of the serotonin transporter in heterozygous (long 3' UTR BDNF: +15% vs SERT$^{+/+}$, p>0.05; exon I: -3% vs SERT$^{+/+}$, p>0.05; exon IV: +38% vs SERT$^{+/+}$, p>0.05) as well as in knock out rats (long 3' UTR BDNF: -6% vs SERT$^{+/+}$, p>0.05; exon I: -17% vs SERT$^{+/+}$, p>0.05; exon IV: +28% vs SERT$^{+/+}$, p>0.05) (Fig.5A-C).

3'UTR long

A

EXON I
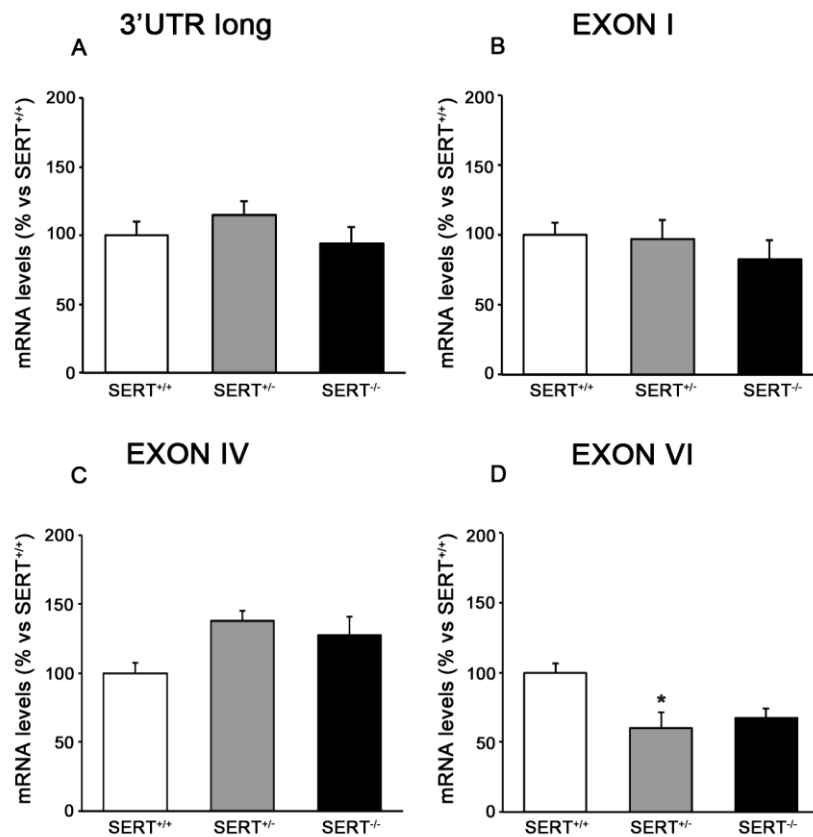
B

EXON IV

C

EXON VI

D

Fig.5 Deletion of SERT gene modulates long 3'UTR BDNF, exon I, exon IV and exon VI gene expression in the dorsal hippocampus.
Long 3'UTR BDNF (A), exon I (B), exon IV (C) and exon VI (D) mRNA levels were measured in the dorsal hippocampus of SERT^{+/-} and SERT^{-/-} rats, as compared to their wild-type (SERT^{+/+}) counterparts. The data, expressed as a percentage of +/+ animals (set at 100%), are the mean±SEM from 6 to 8 independent determinations. *$p<0.05$ vs SERT^{+/+} rats (one-way ANOVA with Bonferroni post hoc test).

Finally in the prefrontal cortex we found a reduction of BDNF exon I and IV mRNA levels in both heterozygous (exon I: -15% vs SERT^{+/+}, $p<0.01$; exon IV: -18% vs SERT^{+/+}, $p<0.05$) and SERT knock out rats (exon I: -19% vs SERT^{+/+}, $p<0.001$; exon IV: -33% vs SERT^{+/+}, $p<0.001$) (Fig.6B,C). On the other hand long 3' UTR BDNF transcript and exon VI are reduced only in SERT^{-/-} rats (respectively -25% vs SERT^{+/+}, $p<0.05$; −30% vs SERT^{+/+}, $p<0.001$). (Fig.6A,D).
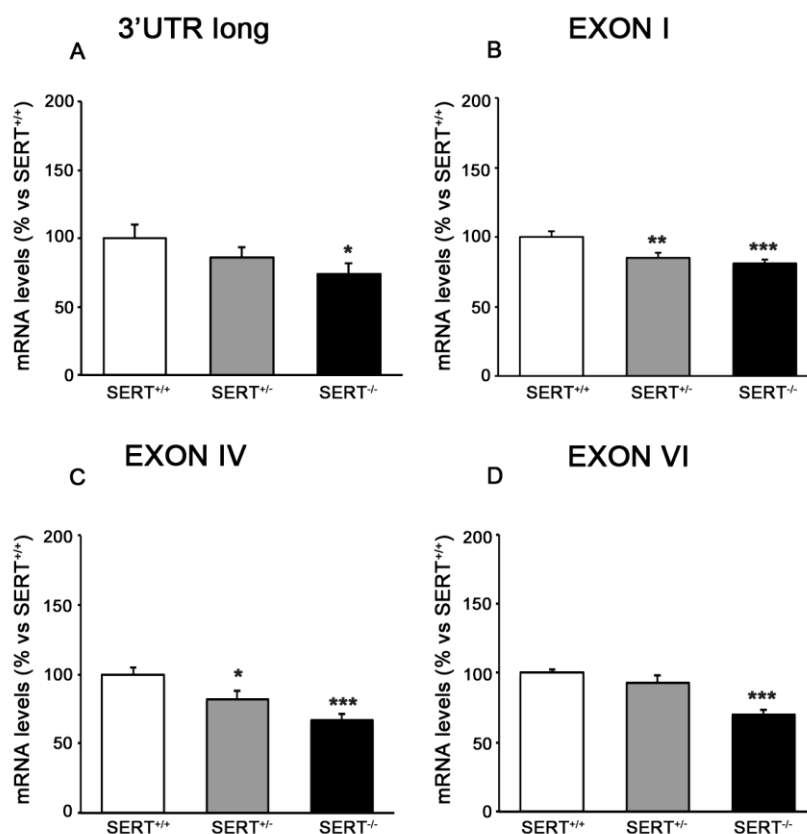
Fig.6 Deletion of SERT gene modulates long 3'UTR BDNF, exon I, exon IV and exon VI gene expression in the prefrontal cortex.

Long 3'UTR BDNF (A), exon I (B), exon IV (C) and exon VI (D) mRNA levels were measured in the prefrontal cortex of SERT^{+/-} and SERT^{-/-} rats, as compared to their wild-type (SERT^{+/+}) counterparts. The data, expressed as a percentage of +/+ animals (set at 100%), are the mean±SEM from 7 to 14 independent determinations. *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs SERT^{+/+} rats (one-way ANOVA with Bonferroni post hoc test).

In order to evaluate if the modulation of BDNF gene expression observed in the ventral hippocampus, dorsal hippocampus and in the prefrontal cortex of SERT mutant rats was paralleled by similar effects at translational level, we performed western blot analysis of the crude synaptosomal fraction from these animals. We found that in the ventral hippocampus (Fig.7A,B) the precursor form of the neurotrophin (proBDNF) is not modulated in SERT^{-/-} rats, while the mature BDNF is significantly reduced in mutant rats (-34% vs SERT^{+/+}, $p<0.05$). Conversely in the dorsal hippocampus (Fig.7C,D), proBDNF is reduced in SERT^{-/-} animals (-33%, vs SERT^{+/+}, $p<0.05$) and mBDNF protein levels are slightly but not significantly affected by lack of the serotonin transporter (-36% vs SERT^{+/+}, $p>0.05$).

Similarly to the dorsal hippocampus, the levels for mature BDNF were unchanged at cortical level (Fig.7E,F) (-6% vs SERT$^{+/+}$, p>0.05), while the levels of the precursor form were significantly reduced in SERT$^{-/-}$ rats (−31% vs SERT$^{+/+}$, p<0.01).
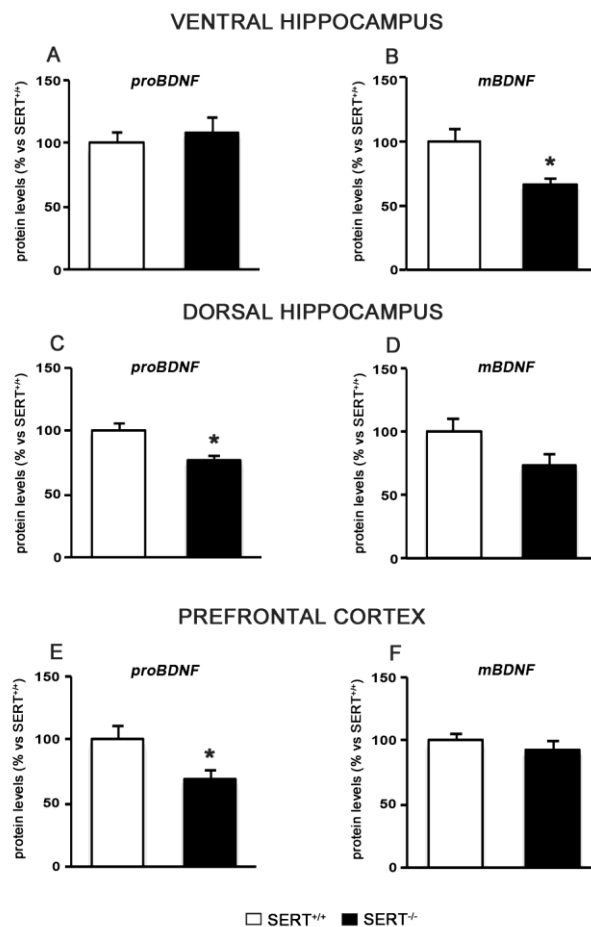

Fig.7 Altered levels of BDNF protein in the ventral hippocampus, in the dorsal hippocampus and in the prefrontal cortex of SERT mutant rats.
The precursor of the neurotrophin (proBDNF) (A,C,E) and its mature form (mBDNF) (B,D,F) were measured in the crude synaptosomal fraction of ventral hippocampus (A,B), dorsal hippocampus (C,D) and in the prefrontal cortex (E,F) of SERT$^{-/-}$ rats, as compared to their wild-type (SERT$^{+/+}$) counterparts. The data, expressed as a percentage of +/+ animals (set at 100%), are the mean±SEM from 6 to 14 independent determinations. *p<0.05 vs SERT$^{+/+}$ rats (Student t-test).

The reduction of BDNF expression observed in SERT mutant rats might be due to the lack of the transporter during the entire life, encompassing development as well as adulthood. To discriminate between these possibilities, we have used a pharmacological strategy and we investigated the neurotrophin expression in the prefrontal cortex after subchronic (21 days) treatment of adult wild type and mutant rats with fluoxetine, a selective-serotonin reuptake inhibitor. Indeed, as shown in Fig.8, the antidepressant significantly increased total BDNF mRNA levels in wild type

rats (+32% vs SERT$^{+/+}$/veh, p<0.01). Conversely, the reduced neurotrophin expression in vehicle-injected SERT$^{-/-}$ rats (−40% vs SERT$^{+/+}$/veh, p<0.001) was not significantly affected by fluoxetine administration (-50% vs SERT$^{+/+}$/flx, p<0.001). These results suggest that prolonged blockade of SERT at adulthood is not sufficient to determine the reduction of BDNF expression observed in SERT$^{-/-}$ rats. Moreover, the lack of fluoxetine effect in SERT mutant rats confirms that these animals do not express a functional serotonin transporter.

Since impairment of SERT in adulthood does not appear to be responsible for BDNF down-regulation in the prefrontal cortex, it is feasible to hypothesize that the reduction of the neurotrophin may represent a long-lasting consequence of transporter dysfunction during development, in agreement with what has been shown for the behavioural phenotype of SERT$^{-/-}$ mice (Ansorge et al., 2004).
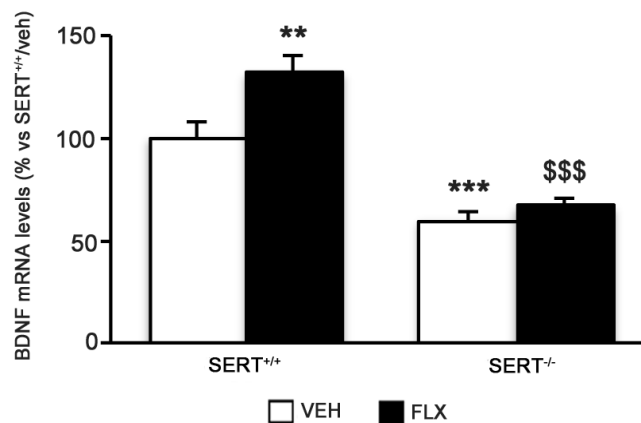

Fig.8 Chronic fluoxetine treatment increases total BDNF mRNA levels in prefrontal cortex of adult wild-type rats but not in SERT$^{-/-}$ rats.
SERT$^{+/+}$ and SERT$^{-/-}$ rats received daily injection of the selective-serotonin reuptake inhibitor fluoxetine (10 mg/kg i.p.) or vehicle for 3 weeks (n=8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

4.2 Developmental influence of the serotonin transporter on the expression of the neurotrophin BDNF.

The serotonin transporter is expressed much more broadly during development than in adulthood and it is conceivable that perturbation of SERT expression and function during critical periods early in development might lead to detrimental consequences later in life. Moreover SERT is arguably the predominant mechanism controlling the strength and duration of serotonergic neurotransmission, which plays a particularly salient role as a developmental signal, important for the proper wiring of neural circuits (Levitt et al., 1983; Gaspar et al., 2003). Therefore, in order to identify when the neuroplastic alteration of SERT$^{-/-}$ animals are established, we investigated the gene expression of BDNF in different brain region of SERT knockout rats at different ages of life. Given the complex genomic structure of the neurotrophin (Pruunsild et al., 2011), we also investigated the influence of the lack of SERT during development on different BDNF transcripts.

First, we investigated the levels of total BDNF mRNA levels in the ventral and the dorsal hippocampus and in prefrontal cortex, and we observed an overall reduction of the neurotrophin gene expression in SERT mutant rats.

Specifically, in the ventral hippocampus (Fig.9A), even if a decrease of BDNF expression is detected in the SERT$^{-/-}$ rats compared to SERT$^{+/+}$ already at birth (PND0: -23% vs SERT$^{+/+}$, $p>0.05$), the effect became statistically significant starting from PND 14 (-25% vs SERT$^{+/+}$, $p<0.05$) and became more considerable at PND 21 (-41% vs SERT$^{+/+}$, $p<0.001$) and PND 28 (-50% vs SERT$^{+/+}$, $p<0.001$). This is probably due to the fact that in the mutant rats BDNF mRNA levels remain relatively constant from the second week of life, whereas there is a progressive increase in SERT$^{+/+}$ rats.

In the dorsal hippocampus (Fig.9B) the BDNF transcript levels are strongly reduced in SERT$^{-/-}$ animals at birth (PND0: -43% vs SERT$^{+/+}$, $p<0.01$) and remain reduced in mutant rats during the first four weeks of life (PND7: -39% vs SERT$^{+/+}$, $p<0.05$; PND14: -32% vs SERT$^{+/+}$, $p<0.05$; PND21: -32% vs SERT$^{+/+}$, $p<0.05$; PND28: -32% vs SERT$^{+/+}$, $p<0.05$).

As shown in Fig.9C, in the prefrontal cortex BDNF mRNA levels increased 7-fold in wild-type animals between PND7 and PND14 while, interestingly, in the mutant rats the reduction of the neurotrophin gene expression due to the lack of the SERT is established in the same time window since it begins at PND7 (-38% vs SERT$^{+/+}$, p<0.01) and remained stable in the subsequent post natal days (PND14: -46% vs SERT$^{+/+}$, p<0.001; PND21: -40% vs SERT$^{+/+}$, p<0.001; PND28: -27% vs SERT$^{+/+}$, p<0.001).
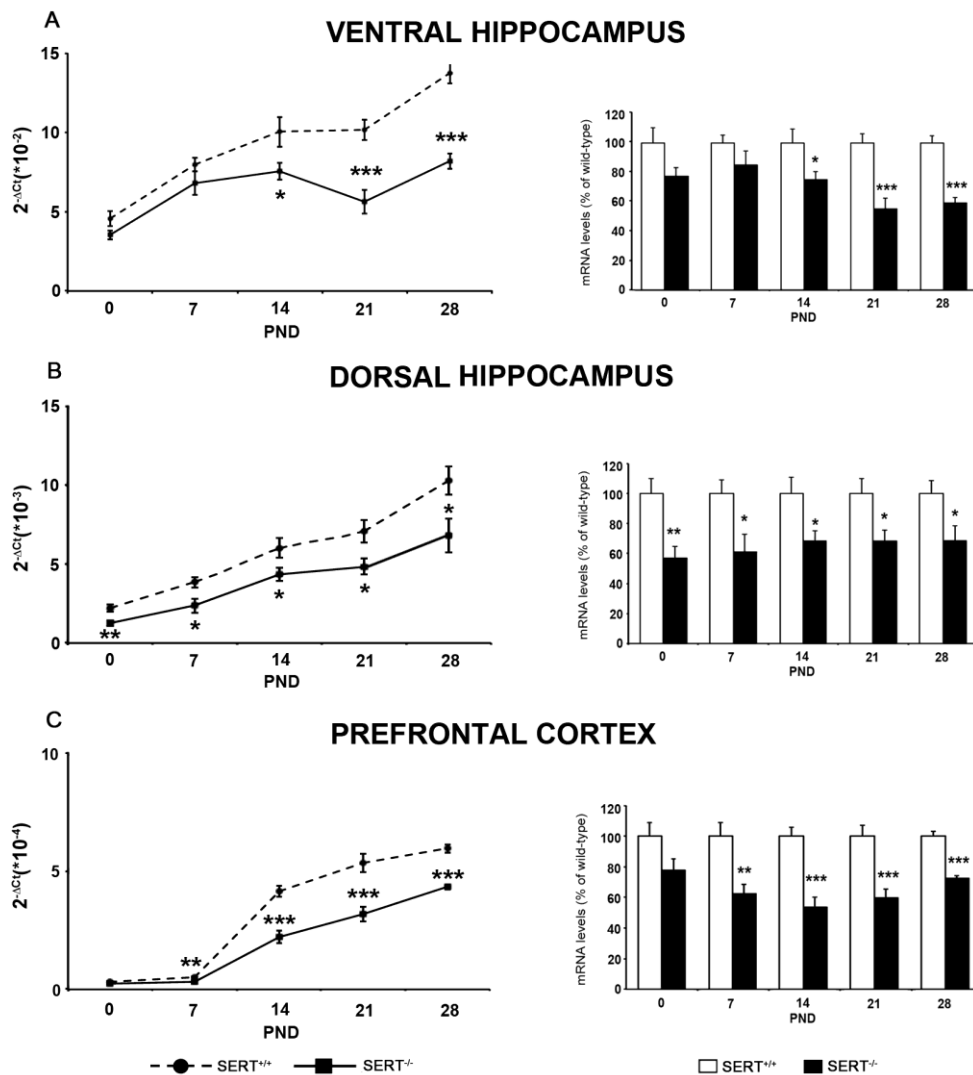

Fig.9 Developmental analysis of BDNF expression changes in SERT$^{-/-}$ rats.
BDNF mRNA levels were measured in the ventral hippocampus (A), in the dorsal hippocampus (B) and in the prefrontal cortex (C) of SERT$^{-/-}$ and SERT$^{+/+}$ rats at postnatal days (PND) 0, 7, 14, 21, 28. The data, expressed as fold change (where ΔCt is the difference between the threshold cycle of the target gene and the housekeeping gene) and as a percentage of wild-type animals at each PND (set at 100%), are the mean ± SEM of 6-12 independent determinations. *p<0.05, **p<0.01, ***p<0.001 vs SERT$^{+/+}$ rats (Student t-test).

We next investigated the expression levels of the long 3' UTR BDNF and of the exons I, IV and VI in the three areas.

In the ventral hippocampus, the mRNA levels of long 3'UTR BDNF and exon VI (Fig. 10A,D) show a similar pattern of expression that also parallels the changes observed for the total form of the neurotrophin. Moreover, the trend is the same between SERT$^{+/+}$ and SERT$^{-/-}$, but both the transcripts mRNA levels are decreased already from birth in the mutant rats.

Conversely, no difference in the expression levels of exons I and IV (Fig.10B,C) are observed between SERT$^{-/-}$ and SERT$^{+/+}$ at PND0. In wild type rats the expression of both the transcripts strongly increased from birth until PND14 and remained constant thereafter. On the contrary, in SERT$^{-/-}$ animals, starting from PND7 we observed a significant decrease of exon I expression, effect that remained similar at PND14, and became slightly more pronounced in the following time points analysed. Differently, BDNF exon IV mRNA levels are fully comparable between SERT$^{+/+}$ and SERT$^{-/-}$ until PND 14, whereas starting from PND21 we found a statistically significant reduction. Notably, the mRNA levels of exon IV in the wild type increased up PND 14 and then remain stable whereas, after this age, in the mutant rats we observed a decline of exon IV expression.
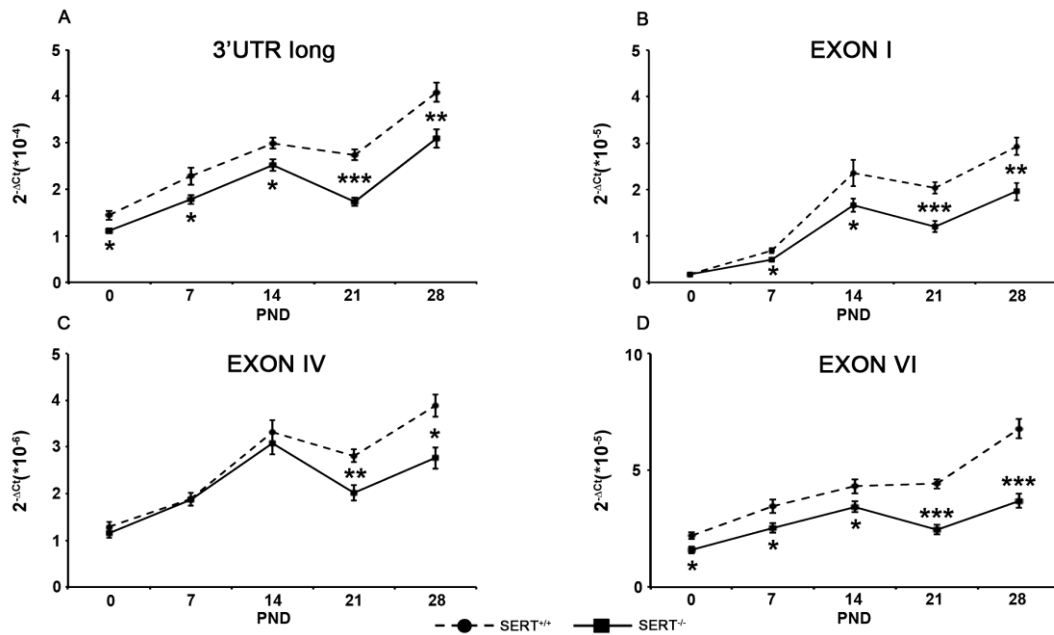
Fig.10 Developmental analysis of BDNF isoforms expression changes in the ventral hippocampus of SERT$^{-/-}$ rats.
Long 3'UTR BDNF (A), exon I (B), exon IV (C) and exon VI (D) mRNA levels were measured in the ventral hippocampus of SERT$^{+/+}$ and SERT$^{-/-}$ rats at postnatal days (PND) 0, 7, 14, 21, 28. The data, expressed as fold change (where ΔCt is the difference between the threshold cycle of the target gene and the housekeeping gene), are the mean ± SEM of 6-12 independent determinations. *p<0.05, **p<0.01, ***p<0.001 vs SERT$^{+/+}$ rats (Student t-test).

In the dorsal hippocampus we found a similar modulation of long 3'UTR BDNF and exon VI in SERT$^{-/-}$ rats. Specifically long 3'UTR BDNF transcript is significantly reduced starting from PND14, while exon VI from PND21 and their reduction remains stable until PND28 (Fig.11A,D). Exon IV expression is decreased in SERT$^{-/-}$ animals since PND0 and the reduction remains stable in the subsequent post natal days (Fig.11C). Conversely we did not found any reduction of exon I mRNA levels between SERT$^{-/-}$ and SERT$^{+/+}$, with the exception of PND7 in which the neurotrophin levels are slightly but significantly reduced in mutant rats (Fig.11B).
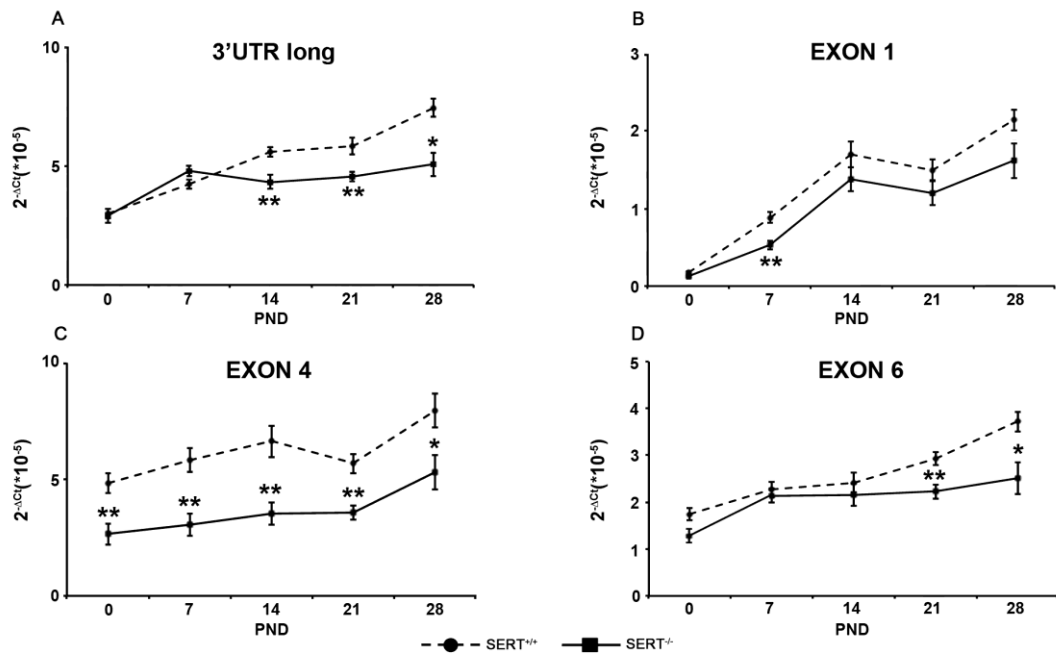
Fig.11 Developmental analysis of BDNF isoforms expression changes in the dorsal hippocampus of SERT$^{-/-}$ rats.
Long 3'UTR BDNF (A), exon I (B), exon IV (C) and exon VI (D) mRNA levels were measured in the dorsal hippocampus of SERT$^{+/+}$ and SERT$^{-/-}$ rats at postnatal days (PND) 0, 7, 14, 21, 28. The data, expressed as fold change (where ΔCt is the difference between the threshold cycle of the target gene and the housekeeping gene), are the mean $\pm$ SEM of 6-12 independent determinations.
*$p<0.05$, **$p<0.01$ vs SERT$^{+/+}$ rats (Student t-test).

In the prefrontal cortex (Fig.12A-D) we found a homogenous pattern of modulation for all the transcripts measured through the different periods of life that also parallels total BDNF mRNA expression. Indeed, no changes are observed at PND0, whereas from postnatal day 7 all the isoforms analysed appear to be reduced in SERT$^{-/-}$ compared to SERT$^{+/+}$. Interestingly, in SERT$^{+/+}$ rats the expression of all the exons examined underwent a sharp increase from PND7 to PND14, an effect that was smaller in SERT$^{-/-}$ rats, thus determining the difference that remains relatively stable thereafter.
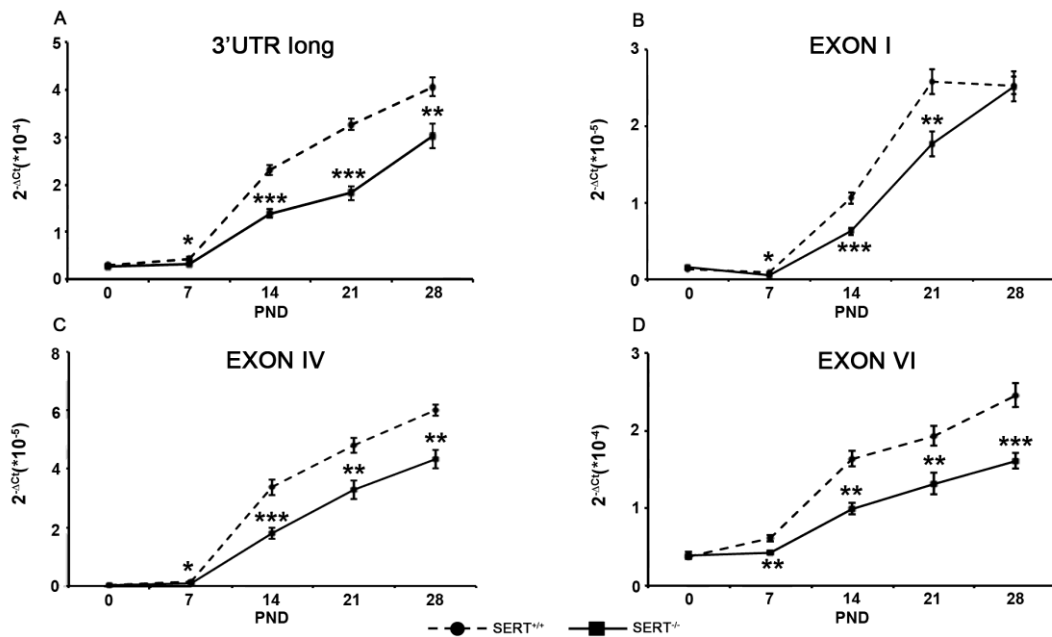
Fig.12 Developmental analysis of BDNF isoforms expression changes in the prefrontal cortex of SERT$^{-/-}$ rats.
Long 3'UTR BDNF (A), exon I (B), exon IV (C) and exon VI (D) mRNA levels were measured in the prefrontal cortex of SERT$^{+/+}$ and SERT$^{-/-}$ rats at postnatal days (PND) 0, 7, 14, 21, 28. The data, expressed as fold change (where ΔCt is the difference between the threshold cycle of the target gene and the housekeeping gene), are the mean ± SEM of 6-12 independent determinations.
*p<0.05, **p<0.01, ***p<0.001 vs SERT$^{+/+}$ rats (Student t-test).

In order to investigate if the reduction of BDNF mRNA levels was associated to similar changes at translational levels, we measured the levels of proBDNF and mBDNF in the crude synaptosomal fraction, which may represent a ready-to-use pool of the neurotrophin (Poo, 2001). We decided to perform the analysis in the ventral hippocampus and in the prefrontal cortex at postnatal days 7 and 21, since at PND7 we found a partial decrease of the neurotrophin, while at PND21 the reduction of BDNF is fully established.

In the ventral hippocampus (Fig.13A,B) of PND 7 rats there were no significant differences in the levels of both proBDNF as well as mBDNF (+5% vs SERT$^{+/+}$, p>0.05, -12% vs SERT$^{+/+}$, p>0.05, respectively) between genotypes, whereas at PND21 the levels of mBDNF were significantly reduced in SERT$^{-/-}$ rats as compared to SERT$^{+/+}$ rats (-50% vs SERT$^{+/+}$, p<0.01) (Fig.13D). A reduction was found also for proBDNF even if this effect did not reach statistical significance (-33% vs SERT$^{+/+}$, p>0.05) (Fig.13C).

Similarly, in the prefrontal cortex of PND7 rats (Fig.13E,F) the deletion of the SERT gene did affect the levels of neither proBDNF (-28% vs SERT$^{+/+}$, p>0.05) nor mBDNF

(+12% vs SERT$^{+/+}$, p>0.05) in the crude synaptosomal fraction. Conversely, at PND21 (Fig.13G,H), the levels of mBDNF were significantly reduced in SERT$^{-/-}$ rats compared to SERT$^{+/+}$ rats (-49% vs SERT$^{+/+}$, p<0.05). The alteration of proBDNF, however, did not reach statistical significance (-41% vs SERT$^{+/+}$, p>0.05).
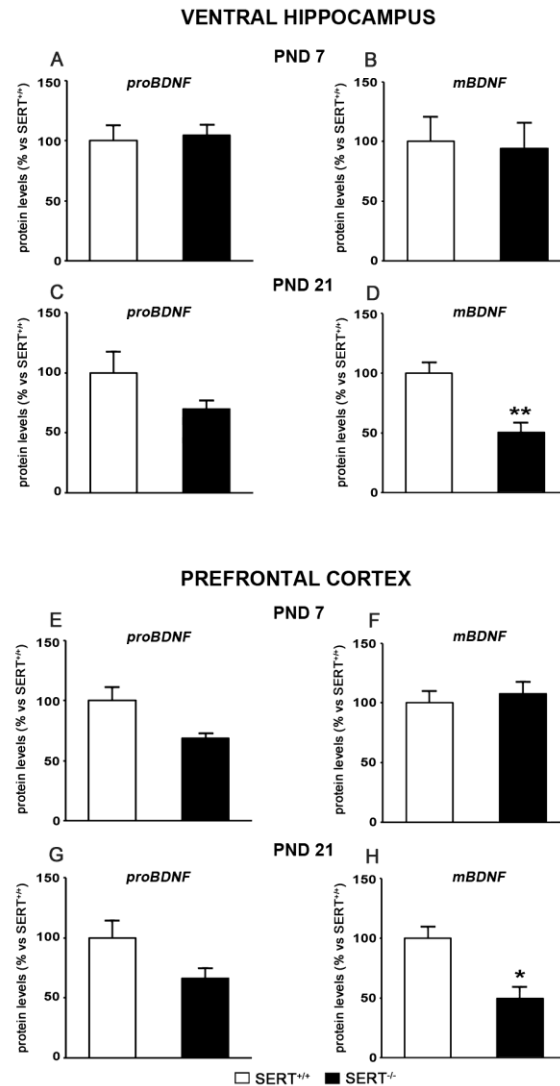

Fig.13 Analysis of BDNF protein levels in the SERT$^{-/-}$ rats during development.
The protein levels of the precursor (A, C, E, G) and the mature (B, D, F, H) forms of BDNF were measured in the ventral hippocampus (A, B, C, D) and in the prefrontal cortex (E, F, G, H) of SERT$^{+/+}$ and SERT$^{-/-}$ rats at postnatal days (PND) 7 (A, B, E, F) and 21 (C, D, G, H). The data, expressed as a percentage of wild type (set at 100%), are the mean ± SEM of 5-10 independent determinations.
*p<0.05, **p<0.01 vs. SERT$^{+/+}$ rats (Student t-test).

4.3 Disruption of SERT affects the expression levels of transcription factors regulating BDNF.

As mentioned before, the BDNF gene has a very complex structure that gives rise to multiple transcripts (Aid et al, 2007; Greer and Greenberg, 2008; Pruunsild et al., 2011). Among the various mechanisms that might potentially underlie the difference of the BDNF expression between SERT$^{+/+}$ and SERT$^{-/-}$ rats, we investigated the expression of the transcription factors Npas4, CREB and CaRF, which cooperate and interact to regulate the transcription of the exon IV (Pruunsild et al., 2011) which is the most abundant and well-characterized BDNF transcript.
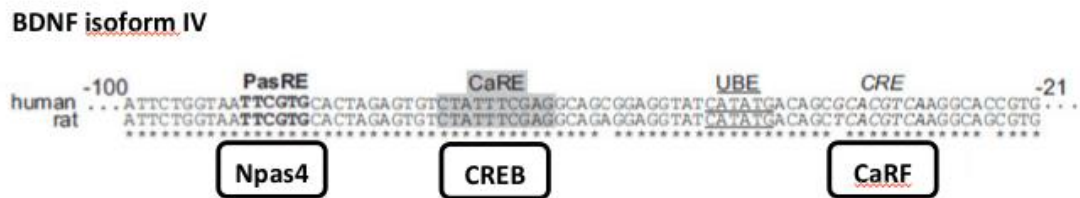
Fig.14 Alignment of the region responsible for neuronal activity-dependent induction of BDNF exon IV in human and rat. PasRE, CaRE and CRE designate the response elements for the respective transcription factors Npas4, CREB and CaRF.

In line with the modulation of BDNF transcript IV, in the ventral hippocampus, we found a significant reduction of Npas4 (-28% vs SERT$^{+/+}$, p<0.01), CREB (-36% vs SERT$^{+/+}$, p<0.01) and CaRF (-44% vs SERT$^{+/+}$, p<0.01) in adult SERT$^{-/-}$ rats compared to wild type animals (Fig.15).

Fig.15 Deletion of SERT gene modulates transcription factors gene expression in ventral hippocampus of SERT$^{-/-}$ rats.
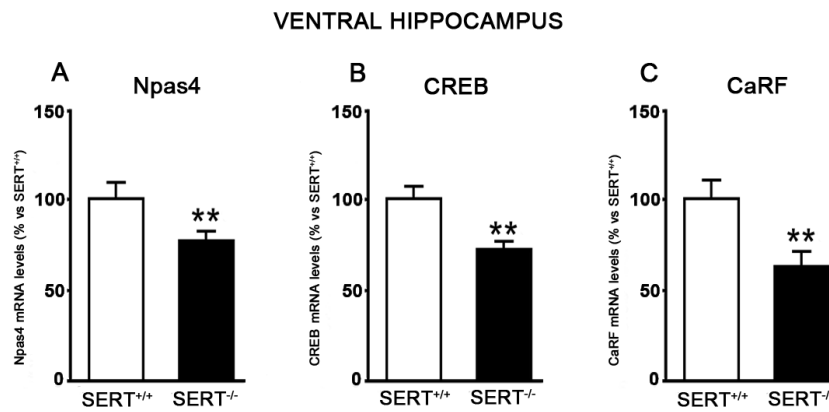The mRNA levels of neuronal PAS domain protein 4 (Npas4) (A), cAMP response element-binding (CREB) (B) and Calcium-Response Factor (CaRF) (C) were measured in the ventral hippocampus of SERT$^{-/-}$ rats, as compared to their wild-type (+/+) counterparts. The data, expressed as a percentage of +/+ animals (set at 100%), are the mean±SEM from 6 to 10 independent determinations.
**p<0.01 vs SERT$^{+/+}$ rats (Student t-test).

In the dorsal hippocampus (Fig.16) we found that only Npas4 is significantly reduced in SERT$^{-/-}$ animals (-27% vs SERT$^{+/+}$, p<0,01), while the mRNA levels of CREB and CaRF are not modulated by the lack of SERT (respectively -18% vs SERT$^{+/+}$, p>0.05; -26% vs SERT$^{+/+}$, p>0.05).

DORSAL HIPPOCAMPUS

Fig.16 Deletion of SERT gene modulates transcription factors gene expression in dorsal hippocampus of SERT$^{-/-}$ rats.
The mRNA levels of neuronal PAS domain protein 4 (Npas4) (A), cAMP response element-binding (CREB) (B) and Calcium-Response Factor (CaRF) (C) were measured in the dorsal hippocampus of SERT$^{-/-}$ rats, as compared to their wild-type (+/+) counterparts. The data, expressed as a percentage of +/+ animals (set at 100%), are the mean±SEM from 6 to 10 independent determinations.
**p<0.01 vs SERT$^{+/+}$ rats (Student t-test).

Finally, as shown in Fig.17, both Npas4 (-45%, $p<0.001$ vs SERT$^{+/+}$) and CaRF (-27%, $p<0.05$, vs SERT$^{+/+}$) expression is reduced in the prefrontal cortex of SERT$^{-/-}$ animals, suggesting that alterations in the expression and function of these transcription factors may sustain the reduction of exon IV mRNA levels observed at adulthood. Conversely SERT disruption does not affect CREB mRNA levels (+7% vs SERT$^{+/+}$, $p>0.05$,).
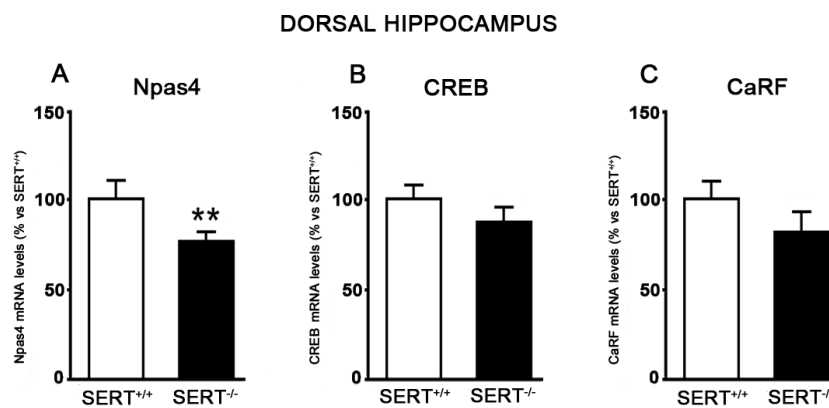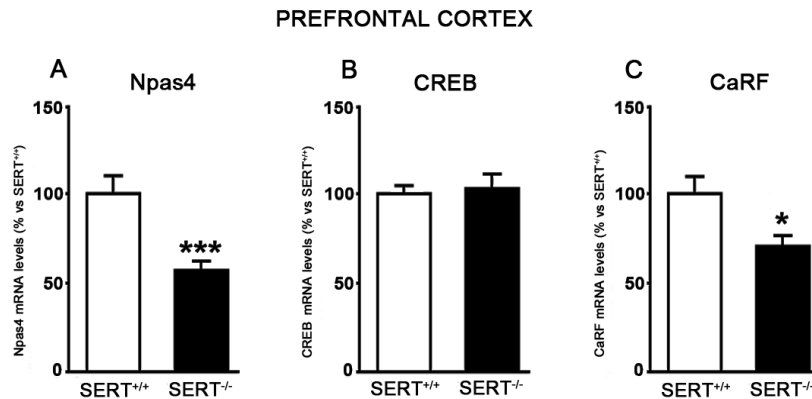

Fig.17 Deletion of SERT gene modulates transcription factors gene expression in the prefrontal cortex of SERT$^{-/-}$ rats.
The mRNA levels of neuronal PAS domain protein 4 (Npas4) (A), cAMP response element-binding (CREB) (B) and Calcium-Response Factor (CaRF) (C) were measured in the prefrontal cortex of SERT$^{-/-}$ rats, as compared to their wild-type (+/+) counterparts. The data, expressed as a percentage of +/+ animals (set at 100%), are the mean±SEM from 6 to 10 independent determinations.
*$p<0.05$, ***$p<0.001$ vs SERT$^{+/+}$ rats (Student t-test).

4.4 Animals with a deletion of SERT gene show impaired expression of GABAergic markers.

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$_A$ γ2) and GABA-β2/3 receptors, and of GABA-producing enzymes glutamic acid decarboxylase (Gad)65 and Gad67 (Lin et al, 2008). Moreover 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, which is altered in SERT$^{-/-}$ rats, may eventually link alterations of BDNF, with an impaired function of the GABAergic system.

Therefore we investigated the expression of three key elements of the GABAergic synapses: the GABA vesicular transporter Vgat, the GABA-producing enzyme Gad67, and the postsynaptic GABA$_A$ γ2, in the whole hippocampus and prefrontal cortex of SERT$^{-/-}$ rats under basal conditions. We found a significant decrease of Vgat expression levels in the hippocampus (-37% vs SERT$^{+/+}$, p<0.05) (Fig.18A), but not in the prefrontal cortex of SERT$^{-/-}$ rats (+16% vs SERT$^{+/+}$, p>0.05,) (Fig.18F). Gad67 expression levels were significantly reduced in the hippocampus (-29% vs SERT$^{+/+}$, p<0.01) (Fig.18B) and in the prefrontal cortex (-18% vs SERT$^{+/+}$, p<0.05) (Fig.18G) of SERT$^{-/-}$ rats. To have an indication of inhibitory synapse number in the mutant rat, we also investigated the expression of the GABA$_A$ γ2 receptor subunit. In the hippocampus, the receptor expression levels were significantly reduced in SERT$^{-/-}$ rats (-38% vs SERT$^{+/+}$, p<0.05) (Fig.18C). In prefrontal cortex, SERT$^{-/-}$ rats showed a reduction of GABA$_A$ γ2 mRNA levels (-35% vs SERT$^{+/+}$, p<0.05) (Fig.18H) as well. 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% vs SERT$^{+/+}$, p<0.01) (Fig.18D), and to a less extent in the

prefrontal cortex (-25% vs SERT$^{+/+}$, p<0.05) (Fig.18I) of SERT$^{-/-}$ rats. With respect to calbindin, its expression was significantly decreased in the hippocampus (-37% vs SERT$^{+/+}$, p<0.01) (Fig.18E), but not in the prefrontal cortex of SERT$^{-/-}$ rats (-11% vs SERT$^{+/+}$, p>0.05) (Fig.18L).
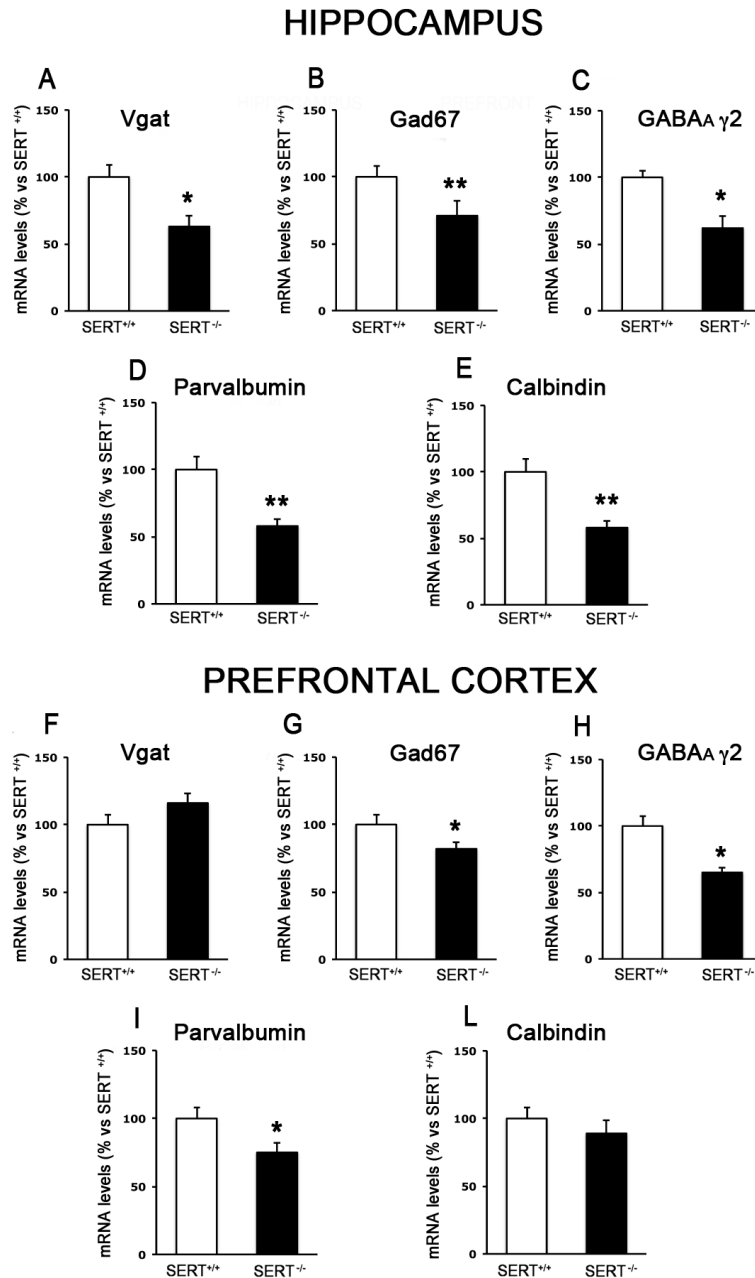
Fig.18 SERT$^{-/-}$ rats show impaired expression of GABAergic markers.
Vesicular transporter (Vgat; A,F), glutamic acid decarboxylase-67 (Gad67; B,G), GABA A receptor, gamma 2 (GABA_A γ2; C,H), Parvalbumin (D,I) and Calbindin (E,L) mRNA levels were measured in the hippocampus (A–E), and prefrontal cortex (F–L) of SERT$^{+/+}$ and SERT$^{-/-}$ rats. The data, expressed as a percentage of +/+ animals (set at 100%), are the mean±SEM from 6 to 10 independent determinations.
*p<0.05, **p<0.01 vs SERT$^{+/+}$ rats (Student t-test).

Given that SERT$^{-/-}$ rats have reduced expression of the neurotrophin Npas4, which has been demonstrated to regulate the GABAergic system, we wanted to assess the possible correlation between the changes in Npas4 gene expression and the alteration in the GABAergic markers in the hippocampus and in the prefrontal cortex. The analyses revealed that, Npas4 mRNA levels correlated positively with the expression of GABA$_A$ γ2 mRNA levels in the hippocampus (r = 0.4120, n=24, p<0.05) (Fig.19A) as well as in the prefrontal cortex (r = 0.5900, n=24, p<0.01) (Fig.19B), suggesting that the lack of the serotonin transporter may be involved in the alteration of the GABAergic system and of the factors regulating it.
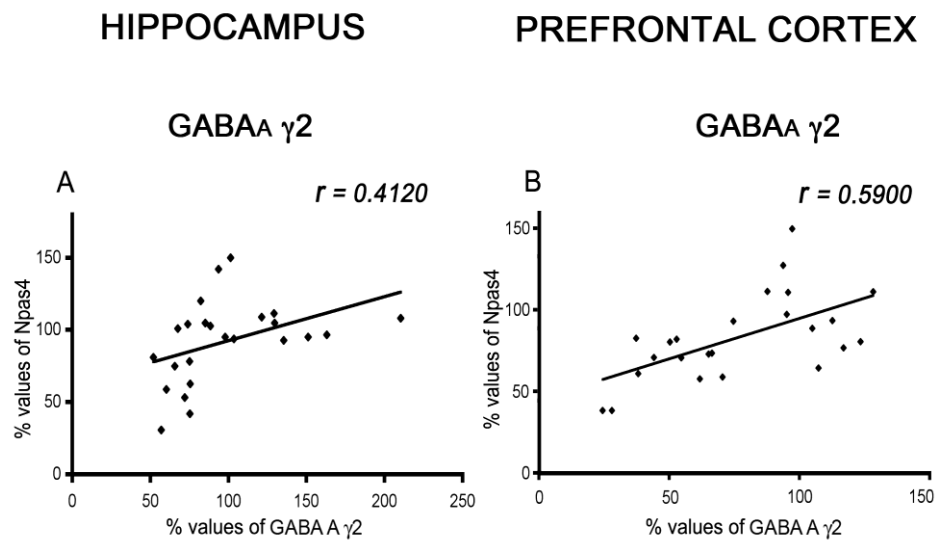
Fig.19 Npas4 mRNA levels correlated positively with the expression of GABA$_A$ γ2.
Correlation analyses between neuronal PAS domain protein 4 (Npas4) and GABA$_A$ receptor, gamma 2 (GABA$_A$ γ2) in the hippocampus (A) and in the prefrontal cortex (B) of SERT$^{+/+}$ and SERT$^{-/-}$ rats. Data points in plots indicate the amount of Npas4, and GABA$_A$ γ2 mRNA levels in single rats. Analyses by Pearson's product–moment correlation (r). The data, from 24 independent determinations, are expressed as a percentage of SERT+ /+/ saline (set at 100%).

4.5 Modulation of BDNF expression in SERT mutant rats after exposure to maternal deprivation.

Stress represents the major precipitating factor in mood disorders. The effects of stress on brain function depend on the timing and duration of the adverse experience. In particular the negative influence exerted by early life events on brain development can be particularly relevant for later psychopathology since stress will impact on structures that are not yet mature (Fumagalli et al., 2008). In order to investigate how the interaction between the SERT genotype and the exposure to an early life stress (ELS) can affect neuroplasticity, wild type and mutant animals were exposed to a maternal deprivation (MD) from PND2 to PND14 (see methods for description).

We performed the analysis of total and long 3'UTR BDNF gene expression in the ventromedial and in the dorsomedial prefrontal cortex of SERT$^{+/-}$ and SERT$^{-/-}$, since the two subregions have different anatomical connectivity and functional implications (Myers-Schulz and Koenigs, 2012).

In the ventromedial prefrontal cortex we observed an overall decrease of the two BDNF transcripts in both genotypes (Fig.20). As shown in figure 20A, total BDNF expression is reduced in heterozygous (-39% vs SERT$^{+/+}$/no stress, p<0.01) and SERT knockout rats (-33% vs SERT$^{+/+}$/no stress, p<0.05). Following exposure to MD, total BDNF mRNA levels are significantly reduced in wild type animals (-46% vs SERT$^{+/+}$/no stress, p<0.001), although the decreased neurotrophin expression in SERT$^{+/-}$ or SERT$^{-/-}$ animals was not exacerbated by exposure to ELS. The pool of neurotrophin transcripts with long 3' UTR underwent changes similar to total BDNF. Indeed their expression is reduced in SERT$^{-/-}$ animals (-40% vs SERT$^{+/+}$/no stress, p<0.05), and slightly but not significantly decreased in SERT$^{+/-}$ rats (-28% vs SERT$^{+/+}$/no stress, p>0.05). The MD paradigm decreases long 3'UTR BDNF in SERT$^{+/+}$ rats (-29% vs SERT$^{+/+}$/no stress, p<0.05), but not in mutant animals (Fig.20B).

In the dorsomedial prefrontal cortex, the profile of BDNF changes was different. Indeed, total mRNA levels (Fig.20C) are not significantly affected in sham SERT$^{+/-}$ or SERT$^{-/-}$ rats, whereas they are significantly increased in SERT$^{+/-}$ rats that were exposed to MD (+34% vs SERT$^{+/-}$/no stress, p<0.05). The expression of long 3' UTR BDNF (Fig.20D) is increased in sham SERT$^{+/-}$ animals (+33% vs SERT$^{+/+}$/no stress, p<0.05) and slightly, but not significantly elevated in SERT$^{-/-}$ rats (+24% vs SERT$^{+/+}$/no stress, p>0.05). However the expression of this pool of BDNF transcripts was not significantly affected by MD in the three genotypes analysed.
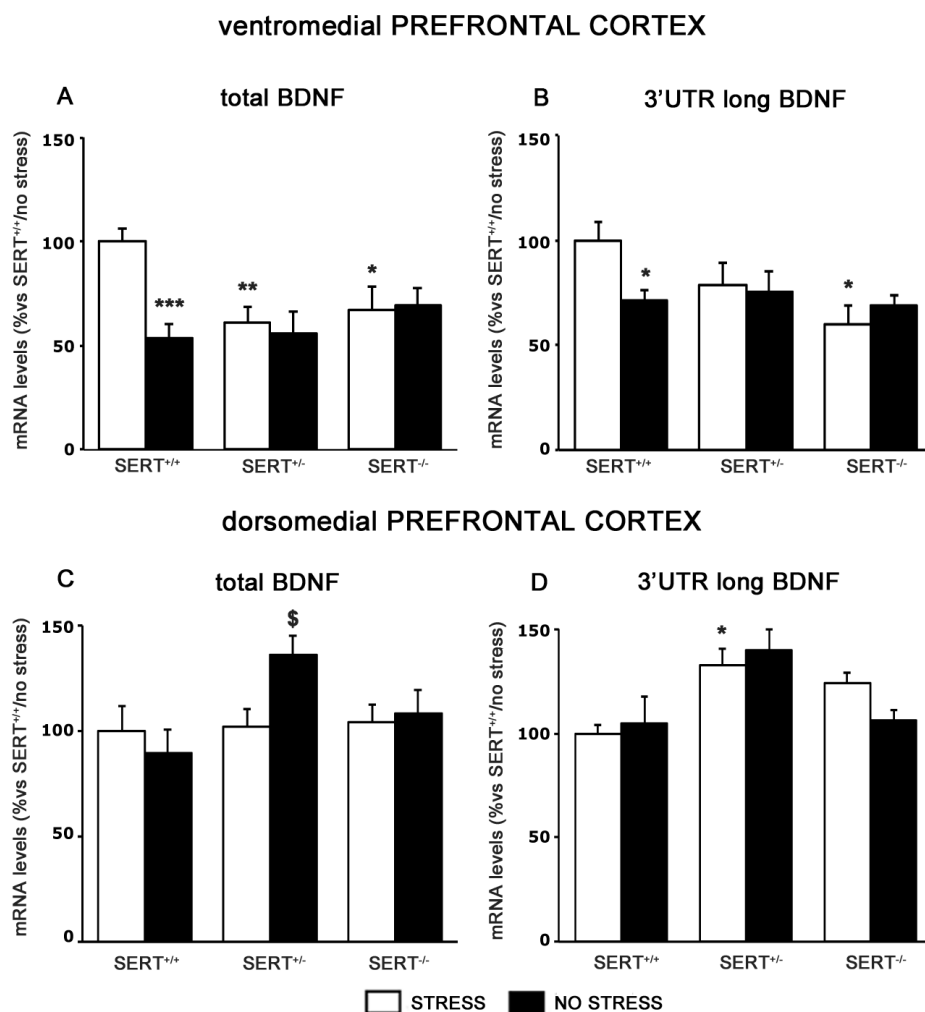
Fig.20 Modulation of total BDNF and long 3'UTR BDNF gene expression in SERT$^{+/-}$ and SERT$^{-/-}$ rats after exposure to stress.
Total BDNF (A,C) and long 3'UTR BDNF (B,D) mRNA levels were measured in ventromedial prefrontal cortex (A,B) and in the dorsomedial prefrontal cortex (C,D) of SERT$^{+/+}$, SERT$^{+/-}$ and SERT$^{-/-}$ rats after exposure to maternal deprivation stress paradigm. The data, expressed as a percentage of SERT$^{+/+}$/no stress animals (set at 100%), are the mean±SEM from 6 to 8 independent determinations.
*p<0.05, **p<0.01, ***p<0.001 vs SERT$^{+/+}$/no stress rats; $p<0.05 vs SERT$^{+/-}$/no stress rats (two-way ANOVA with SCPHT).

4.6 Modulation of BDNF expression by long-term duloxetine treatment in animals with a deletion of the SERT gene.

Since SERT$^{-/-}$ rats show a reduction of total BDNF mRNA levels in the hippocampus and in the prefrontal cortex, we decided to investigate the effect of a long-term treatment with the antidepressant drug duloxetine (a SNRI, dual blocker of the reuptake of the serotonin and norepinephrine) on BDNF expression in SERT$^{-/-}$ rats. Indeed there is a general agreement that antidepressant drug treatment can increase the expression of BDNF in selected brain regions (Berton and Nestler, 2006; Martinowich et al., 2007; Molteni et al., 2010a). In particular we wondered whether the antidepressant treatment was able to normalize the alteration of BDNF detected in this animal model. To this aim, we measured total BDNF mRNA levels in the whole hippocampus and in prefrontal cortex of wild type and SERT$^{-/-}$ rats treated for 21 days with duloxetine.

The mRNA levels of total BDNF are significantly lower in the whole hippocampus of vehicle-injected SERT$^{-/-}$ rats (-12% vs SERT$^{+/+}$/veh, $p<0.05$). Chronic treatment with duloxetine is able to increase total BDNF mRNA levels in wild type (+31% vs SERT$^{+/+}$/veh, $p<0.001$), as well as in SERT$^{-/-}$ rats (+42% vs SERT$^{-/-}$/veh, $p<0.001$) (Fig.21A).

As described above, in the prefrontal cortex we found a significant reduction of total BDNF mRNA levels in SERT$^{-/-}$ rats (-34% vs SERT$^{+/+}$/veh, $p<0.001$). Long-term administration of duloxetine restores the physiological expression of total BDNF in mutant rats (+41% vs SERT$^{-/-}$/veh, $p<0.001$) and significantly increases the mRNA levels of the neurotrophin in SERT$^{+/+}$ animals (+20% vs SERT$^{+/+}$/veh, $p<0.001$) (Fig.21B).
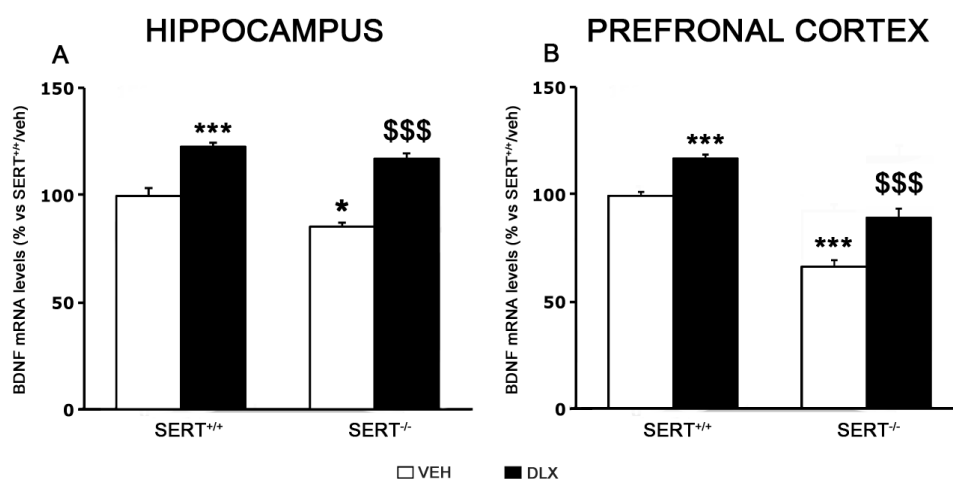
Fig.21 Long-term duloxetine treatment increases total BDNF mRNA levels in SERT$^{+/+}$ and SERT$^{-/-}$ rats.
Total BDNF mRNA levels were measured in hippocampus (A) and prefrontal cortex (B) of SERT$^{+/+}$ and SERT$^{-/-}$ rats treated for 21 days with vehicle (VEH) or duloxetine (DLX) and killed 24 h after the last injection. The data, expressed as a percentage of SERT$^{+/+}$/veh (set at 100%), are the mean±S.E.M. from at least 6 independent determinations.
*$p<0.05$, ***$p<0.001$ vs SERT$^{+/+}$/veh; $$$ $p<0.001$ vs SERT$^{-/-}$/veh (two-way ANOVA with SCPHT).

We next decided to investigate the mRNA levels of major BDNF transcripts in the whole hippocampus and prefrontal cortex of wild type and mutant rats after long-term duloxetine treatment.

Differently from the data of the two distinct hippocampal sub regions, the mRNA levels of long 3'UTR BDNF are significantly reduced in the whole hippocampus of SERT$^{-/-}$ rats (-13% vs SERT$^{+/+}$/veh, $p<0.05$), while BDNF isoform I mRNA levels are not decreased (-8% vs SERT$^{+/+}$/veh, $p>0.05$). Long-term duloxetine treatment is able to increase the expression of the two transcripts in SERT knockout rats (long 3'UTR BDNF: +30% vs SERT$^{-/-}$/veh, $p<0.001$; exon I: +28% vs SERT$^{-/-}$/veh, $p<0.001$) without affecting their levels in wild type animals (long 3'UTR BDNF: +8% vs SERT$^{+/+}$/veh, $p>0.05$; exon I: +9% vs SERT$^{+/+}$/veh, $p>0.05$) (Fig.22A,B).

On the other hand the mRNA levels of BDNF exon IV and VI in the whole hippocampus match the levels found in the two distinct sub regions. In fact the expression of the isoforms IV and VI is reduced in the total hippocampus of SERT$^{-/-}$ rats (respectively -16% vs SERT$^{+/+}$/veh, $p<0.01$; -11% vs SERT$^{+/+}$/veh, $p<0.05$). Chronic treatment with the antidepressant restores the physiological expression of the two transcripts in mutant rats (exon IV: +22% vs SERT$^{-/-}$/veh, $p<0.05$; exon VI: +33% vs

SERT$^{-/-}$/veh, p<0.001), but does not affect their modulation in wild type animals (exon IV: -10% vs SERT$^{+/+}$/veh, p>0.05; exon VI: -4% vs SERT$^{+/+}$/veh, p>0.05) (Fig.22C,D).
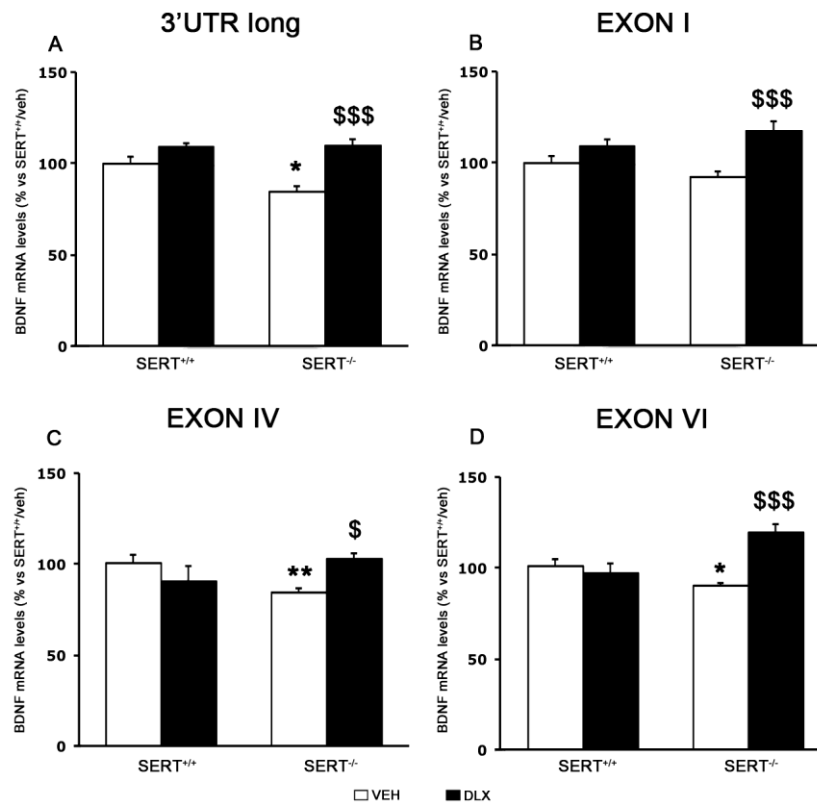

Fig.22 The modulation of BDNF isoforms after long-term duloxetine treatment is influenced by SERT genotype in the hippocampus.
Long 3'UTR BDNF (A), exon I (B), exon IV (C) and exon VI (D) mRNA levels were measured in the hippocampus of SERT$^{+/+}$ and SERT$^{-/-}$ rats treated for 21 days with vehicle (VEH) or duloxetine (DLX) and killed 24 h after the last injection. The data, expressed as a percentage of SERT$^{+/+}$/veh (set at 100%), are the mean±S.E.M. from at least 6 independent determinations.
*p<0.05, **p<0.01 vs SERT$^{+/+}$/veh; $ p<0.05, $$$ p<0.001 vs SERT$^{-/-}$/veh (two-way ANOVA with SCPHT).

As described earlier, the expression levels of the four transcripts are reduced in the prefrontal cortex of mutant rats (Fig.6A-D). Chronic duloxetine treatment increases the mRNA levels of long 3'UTR BDNF and isoforms IV and VI in SERT$^{-/-}$ rats (respectively, +25% vs SERT$^{-/-}$/veh, p<0.05; +51% vs SERT$^{-/-}$/veh, p<0.001 rats; +73% vs SERT$^{-/-}$/veh, p<0.001) but not in SERT$^{+/+}$ animals. Conversely the transcriptional modulation of isoform I seem to be somewhat different from the other transcripts, since duloxetine was able to increase its mRNA levels in wild-type as well as mutant rats (+29% vs SERT$^{+/+}$/veh, p<0.001; +65% vs SERT$^{-/-}$/veh, p<0.001) (Fig.23A-D).
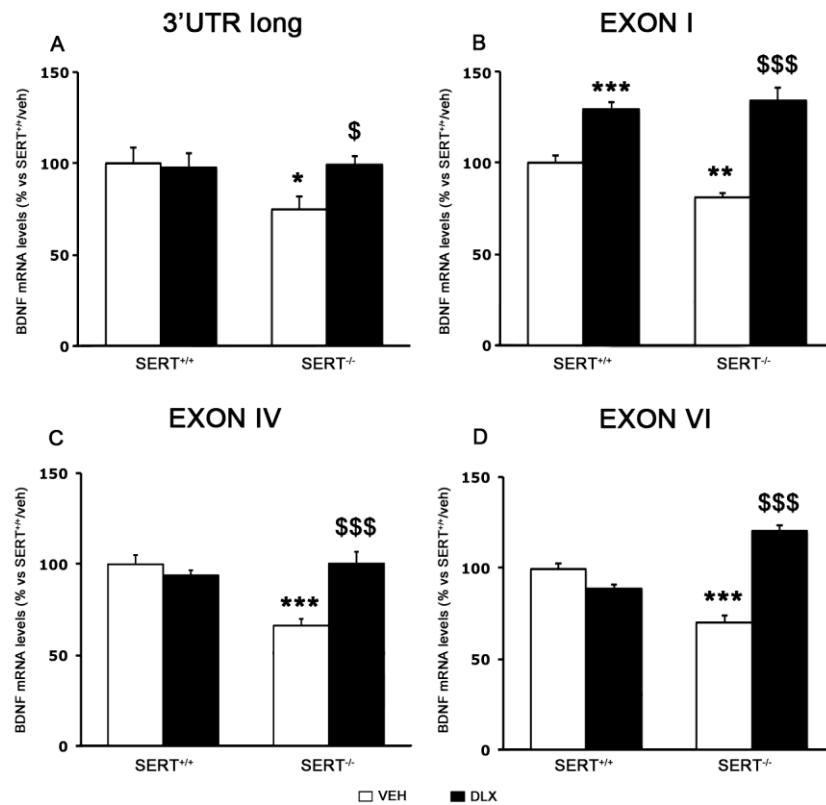
Fig.23 The modulation of BDNF isoforms after long-term duloxetine treatment is influenced by SERT genotype in the prefrontal cortex.

Long 3'UTR BDNF (A), exon I (B), exon IV (C) and exon VI (D) mRNA levels were measured in the prefrontal cortex of SERT$^{+/+}$ and SERT$^{-/-}$ rats treated for 21 days with vehicle (VEH) or duloxetine (DLX) and killed 24 h after the last injection. The data, expressed as a percentage of SERT$^{+/+}$/veh (set at 100%), are the mean±S.E.M. from at least 6 independent determinations.

*p<0.05, **p<0.01, ***p<0.001 vs SERT$^{+/+}$/veh; $ p<0.05, $$$ p<0.001 vs SERT$^{-/-}$/veh (two-way ANOVA with SCPHT).

4.7 Effect of chronic duloxetine treatment on Npas4 and GABAergic markers mRNA levels in SERT$^{-/-}$ rats.

We finally tested whether chronic antidepressant treatment might restore the physiological expression of Npas4 and GABAergic markers in SERT$^{-/-}$ rats. In hippocampus duloxetine normalizes the reduction of Npas4 expression levels in SERT$^{-/-}$ rats (+98% vs SERT$^{-/-}$/veh, p<0.01), but did not influence the mRNA levels of the transcription factor in wild type animals (+9% vs SERT$^{+/+}$/veh, p>0.05) (Fig.24A). Similarly in the prefrontal cortex the reduced levels of Npas4 are normalized after chronic antidepressant treatment in SERT$^{-/-}$ rats (+69% vs SERT$^{-/-}$/veh, p<0.01), while in SERT$^{+/+}$ animals the antidepressant do not affect Npas4 expression (+17% vs SERT$^{+/+}$/veh, p>0.05) (Fig.24B).

Fig.24 Chronic duloxetine treatment modulates the levels of Npas4 gene expression in SERT$^{-/-}$ rats.
Npas4 mRNA levels were measured in the hippocampus (A) and in the prefrontal cortex (B) of SERT$^{+/+}$ and SERT$^{-/-}$ rats treated for 21 days with vehicle (VEH) or duloxetine (DLX), and killed 24 h after the last injection. The data, expressed as a percentage of SERT$^{+/+}$/veh (set at 100%), are the mean±SEM from at least five independent determinations.
***p<0.001 vs SERT$^{+/+}$/veh; $$ p<0.01 vs SERT$^{-/-}$/veh (two-way ANOVA with SCPHT).
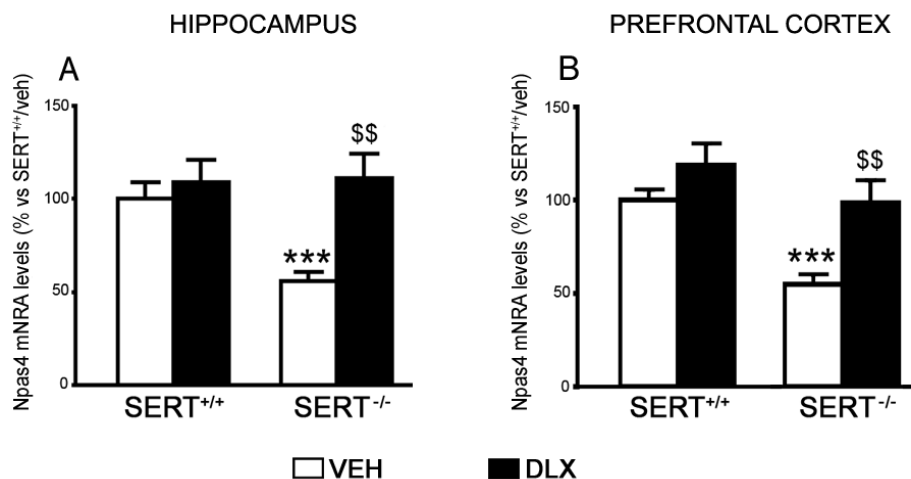
Regarding GABAergic markers we found that long-term duloxetine treatment normalizes the reduction of Vgat (+52% vs SERT$^{-/-}$/veh, p<0.05), Gad67 (+31% vs. SERT$^{-/-}$/veh, p<0.05), and GABA$_A$ γ2 receptor (+80% vs SERT$^{-/-}$/veh, p<0.05) in

hippocampus of SERT$^{-/-}$ rats, without affecting the mRNA levels of the three GABAergic markers in wild type rats (Fig.25A-C).

In the prefrontal cortex duloxetine increases GABA$_A$ γ2 expression in wild type (+45% vs SERT$^{+/+}$/veh, p<0.05) as well as in mutant rats (+91% vs SERT$^{-/-}$/veh, p<0.001) (Fig.25H). However, differently from the hippocampus, the antidepressant treatment was not able to modulate Gad67 (-4%, vs SERT$^{-/-}$/veh, p>0.05) and Vgat (-7% vs SERT$^{-/-}$, p>0.05) mRNA levels in SERT$^{-/-}$ animals (Fig.25F,G).

Duloxetine treatment is also able to modulate parvalbumin and calbindin in the hippocampus and in the prefrontal cortex. Specifically, chronic antidepressant treatment restored parvalbumin (+52% vs SERT$^{-/-}$/veh, p<0.05) and calbindin (+79% vs SERT$^{-/-}$/veh, p<0.01) physiological levels in the hippocampus of SERT$^{-/-}$ rats (Fig.23D,E). Conversely in the prefrontal cortex nor parvalbumin (+19% vs SERT$^{-/-}$/veh, p>0.05), neither calbindin (+8% vs SERT$^{-/-}$/veh, p>0.05) are affected by long-term duloxetine administration (Fig.25I,L).

HIPPOCAMPUS

A Vgat

B Gad67

C GABAA γ2

D Parvalbumin

E Calbindin

PREFRONTAL CORTEX

F Vgat

G Gad67

H GABAA γ2

Parvalbumin

L Calbindin
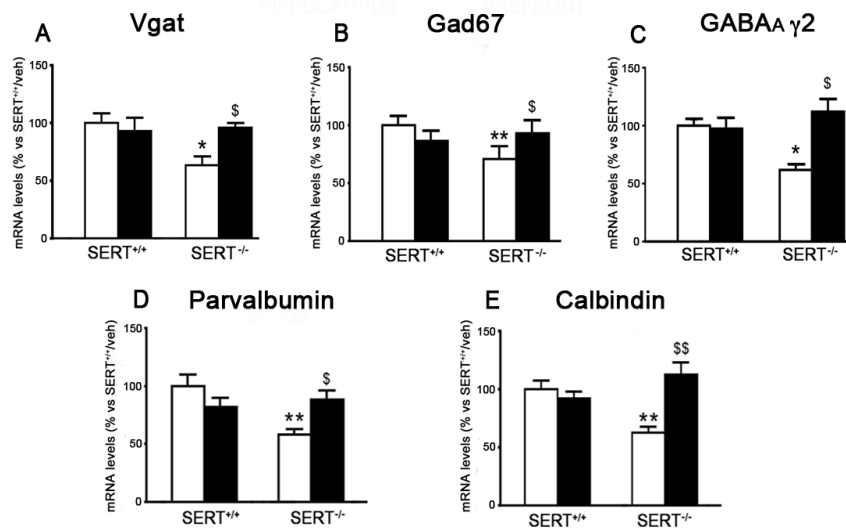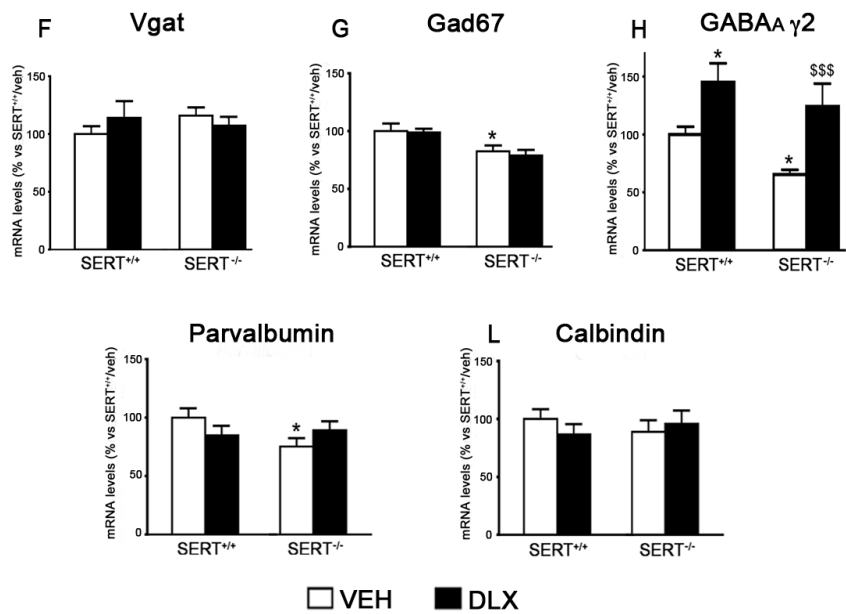
□ VEH ■ DLX

Fig.25 SERT^{-/-} rats show impaired expression of GABAergic markers: modulation by chronic duloxetine treatment.

Vesicular transporter (Vgat; A,F), glutamic acid decarboxylase-67 (Gad67; B,G), GABA A receptor, gamma 2 (GABAA γ2; C,H), Parvalbumin (D,I) and Calbindin (E,L) mRNA levels were measured in the hippocampus (A–E), and prefrontal cortex (F–L) of SERT$^{+/+}$ and SERT$^{-/-}$ rats treated for 21 days with vehicle (VEH) or duloxetine (DLX), and killed 24 h after the last injection. The data, expressed as a percentage of +/+ animals (set at 100%), are the mean±SEM from 6 to 10 independent determinations.

*p<0.05, **p<0.01 vs SERT$^{+/+}$/veh rats: $ p<0.05, $$ p<0.001, $$$ p<0.001 vs SERT$^{-/-}$/veh rats (two-way ANOVA with SCPHT)

5. DISCUSSION

Animals with a target deletion of the serotonin transporter represent a model of anxiety and depressive behavior (Olivier et al., 2008) and can be used to investigate the mechanisms that may underlie such phenotype. In line with the concept that several psychiatric conditions, such as mood disorders, are associated with deficits or impairment of neuronal plasticity (McClung and Nestler, 2008; Pittenger and Duman, 2008, Calabrese et al., 2011), we demonstrate that, under basal condition, adult SERT$^{-/-}$ rats have decreased expression of the neurotrophin BDNF, a major player in neuronal plasticity. We found that SERT$^{-/-}$ rats have reduced BDNF expression in the ventral hippocampus, that specifically modulates emotional and affective processes, and in the prefrontal cortex, but not in the dorsal hippocampus involved in spatial learning and memory function. Interestingly, within the prefrontal cortex a partial deletion of the SERT gene, as occurring in SERT$^{+/-}$ rats, is sufficient to determine a significant reduction of BDNF expression.

The analysis of BDNF different transcripts suggests that the effects observed in the ventral hippocampus of mutant rats are primarily due to a reduction of isoform I and IV, whereas a more generalized decrease was found in the prefrontal cortex of SERT$^{-/-}$ rats. Furthermore in heterozygous rats we found a reduction of exon I in the ventral hippocampus, and of exon I and IV in prefrontal cortex.

Changes in the neurotrophin expression observed in prefrontal cortex and hippocampus of rats with partial deletion of SERT may hold translational implications, since heterozygous SERT rats mimic more closely the human condition of reduced expression of the serotonin transporter in individuals carrying the S allele gene. In line with this possibility, our laboratory has previously demonstrated that BDNF expression is significantly reduced in peripheral cells of control individuals carrying the S allele for the 5-HTTLPR (Molteni et al., 2010a).

The more generalized effect of SERT deletion within the prefrontal cortex may be related to structural alterations in this brain region. Neuroimaging studies have indeed demonstrated that short allele carriers, who have lower SERT expression,

showed reduced gray matter volume in limbic regions (Pezawas et al., 2008).

Moreover, changes in the expression of selected BDNF transcripts, as occurring in SERT mutant rats, may reflect altered function of neurotransmitter systems or circuits involved in the control of BDNF production.

The down-regulation of total BDNF mRNA levels is paralleled by a similar reduction of mature BDNF protein levels in the crude synaptosomal fraction of ventral hippocampus, whereas in the dorsal hippocampus and in the prefrontal cortex only the levels of proBDNF are reduced. These data suggest that despite similar changes at mRNA levels, the regulation of BDNF protein in the ventral hippocampus and the prefrontal cortex can be different.

Deletion of SERT gene in mice (Holmes et al., 2003; Lira et al., 2003) and rats (Olivier et al., 2008) produces anxiety- and depression-related behaviors, an effect that may appear paradoxical given that chronic administration of SSRI antidepressant, which blocks SERT function, results in antidepressant effects. Indeed the anxious and depressed phenotype of SERT^{-/-} mice may represent the long-lasting consequence of impaired SERT function during early development (Ansorge et al., 2008; Ansorge et al., 2004).

As expected, we show that reduced expression of BDNF in SERT^{-/-} rats is not due to adult impairment of the transporter. In fact chronic treatment of adult wild-type rats with the SSRI fluoxetine increased BDNF gene expression rather than reduced it. These findings are in line with data from several laboratories that during the last 10-15 years have clearly shown that repeated treatment with antidepressant drugs up-regulates BDNF expression in different brain structures, an effect that may normalize defective neuronal plasticity in depressed subjects (Calabrese et al., 2007; Duman and Monteggia, 2006; Molteni et al., 2010a).

Moreover SERT blockade from postnatal days 4 to 21 (as produced by fluoxetine treatment) may not be sufficient to determine a long-lasting reduction of BDNF expression, although a transient decrease of the neurotrophin can be observed during the second week of postnatal life (Karpova et al., 2009).

Hence, the reduction of BDNF levels in SERT^{-/-} rats may not be due to early postnatal

impairment of the transporter but may encompass altered SERT function in utero as well as during adolescence.

In order to understand the developmental profile of the neuroplastic alteration in mutant rats, we therefore investigated BDNF expression from birth to post natal day (PND) 28. The present data demonstrate that alterations of BDNF expression following genetic inactivation of SERT originates early in development and worsens during the first 2-3 weeks of postnatal life, suggesting that the maturational profile of the serotonergic system (Daws and Gould, 2011) may interfere with the developmental maturation of the neurotrophin in key brain regions. This may shape the path towards permanent alterations of neuroplasticity in selected brain regions including the hippocampus and the prefrontal cortex.

In particular, the analysis of the developmental profile of the neurotrophin in the ventral hippocampus suggests that, while BDNF mRNA levels increase physiologically between PND0 and PND28 in wild-type animals, its expression reaches a plateau around the end of the second week of life in SERT$^{-/-}$ rats. On the other hand in the dorsal hippocampus BDNF is decreased in SERT$^{-/-}$ rats during the first four weeks. However, the reduction of the neurotrophin seems to have a limited duration, since it normalizes at adulthood. At cortical level, the lack of SERT significantly and markedly affected BDNF expression after the first week of life, a week earlier than in the ventral hippocampus.

In accordance with transcriptional changes, we demonstrated that the protein levels of mature BDNF were decreased in the crude synaptosomal fraction at PND21 whereas limited changes were present at PND7, both in the ventral hippocampus as well as in the prefrontal cortex. Considering the complex BDNF regulation, the reduction of its protein levels in the crude synaptosomal fraction is indicative of deficit in the pool of neurotrophin ready available for release, which is important for synaptic mechanisms and cell-cell communication (Poo, 2001; Calabrese et al., 2007; Molteni et al., 2010a; Lau et al., 2010).

The different modulation of BDNF mRNA levels at adulthood could be the consequences of abnormalities in transcription factors regulating its expression. In

line with this possibility, we demonstrated that total deletion of SERT affect the expression of the transcription factors Npas4, CREB and CaRF, which play a primary role in the transcription of BDNF exon IV, the most abundant BDNF transcript. In agreement with the expression levels of exon IV, we demonstrated that the mRNA levels for all these transcription factors are significantly reduced in the ventral hippocampus. In the dorsal hippocampus we found only the reduction of Npas4, which seems not enough to induce a decrease of transcript IV expression. Conversely, in the prefrontal cortex Npas4 and CaRF are reduced, while CREB levels are unchanged, supporting the hypothesis that CREB may be more involved in the activity-regulated expression of BDNF exon IV, rather than controlling its levels under resting conditions (Hong et al., 2008).

The different profile of BDNF expression in SERT$^{-/-}$ rats may also be the consequence of other systems controlling the regulation of the neurotrophin such as epigenetic mechanisms. In particular, it has been our laboratory that has previously demonstrated an increased DNA methylation in the promoter region of exon VI, which can correlate with the repression of its transcription in the hippocampus of SERT$^{-/-}$ rats. Conversely, the downregulation of BDNF exon IV mRNA levels is associated with reduced acetylation of histone H3, a mechanism driving transcription activation (Molteni et al., 2010a).

One system lying downstream from Npas4 and BDNF that may be relevant for the phenotype of SERT mutants is GABA. Indeed, Npas4 regulates the development of GABAergic synapses, since Npas4 gene silencing 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). We demonstrate that SERT$^{-/-}$ rats show a strong impairment of the GABAergic system in the whole hippocampus as well as in prefrontal cortex, although some differences 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$_A$-γ2 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. 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 (Sanacora et al., 1999; Benes and Berretta, 2001; Krystal et al., 2002; Brambilla et al., 2003; Luscher et al., 2011). In fact, GABAA 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 (Kasa et al., 1982; Gerner et al., 1984; Petty et al., 1992). 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).

In addition to the genetic component, stressful experiences can exacerbate or precipitate depressive disorder. Adverse events early in life can be particularly relevant for later psychopathology since they will impact on structures that are not fully mature. We demonstrated that exposure to the maternal deprivation paradigm reduce the expression of both total and long 3'UTR BDNF transcripts in the ventromedial prefrontal cortex of wild type animals, without exacerbating the already reduced mRNA levels of the neurotrophin in SERT$^{+/-}$ and SERT$^{-/-}$ rats. The present results suggest that within the ventromedial prefrontal cortex BDNF expression is similarly reduced by SERT deletion or exposure to ELS, which can independently produce long-lasting depressive-like behaviour (Holmes et al., 2003; Fumagalli et al., 2007; Olivier et al., 2008). However the combination of MD with the susceptible genotype does not lead to a worsening of the neuroplastic phenotype (BDNF expression). This may suggest that there is a floor effect in the regulation of BDNF expression, which will render difficult the identification for the gene * environment interaction. Future studies will try to establish if milder or shorter stress paradigms could be employed in order to address this important issue, which holds

high translational relevance.

In the dorsomedial prefrontal cortex, involved in cognition and memory function (Dalley et al., 2004) we found a complete different modulation of the neurotrophin. BDNF mRNA levels are not modulated by stress in wild type or knockout rats. Heterozygous animals, on the other hand, show increased expression of long 3'UTR transcripts, while MD can increase total BDNF expression only in SERT$^{+/-}$ rats. This suggests that there are anatomically-specific changes of BDNF expression, which may subserve different functions. For example, recent findings demonstrated that humans and nonhuman primates carrying the S variant of the serotonin transporter outperform subjects carrying the long allele in a wide array of cognitive tasks and show increased social conformity (Homberg and Lesch, 2011).

Finally, we show that chronic treatment with the antidepressant duloxetine is able to normalize BDNF mRNA levels, which are reduced in the whole hippocampus and prefrontal cortex of SERT$^{-/-}$ rats. As demonstrated previously (Calabrese et al., 2007; Molteni et al., 2010a), long-term duloxetine treatment increases total BDNF mRNA levels in the hippocampus and in the prefrontal cortex of wild-type animals.

Moreover, we demonstrate that the mRNA levels of the long 3' UTR BDNF and of transcripts I, IV and VI are normalized after duloxetine treatment in SERT knockout rats. This modulation suggest, that higher levels of long 3' UTR BDNF mRNA may enhance the synaptic pool of BDNF transcripts that, upon stimuli, may undergo local translation (An et al., 2008). Moreover the modulation of isoforms I, IV and VI might represent a specific mechanism through which antidepressant drugs, such as duloxetine, can restore defective plasticity in a vulnerable genotype.

Chronic duloxetine treatment is also able to 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. As a consequence of BDNF and Npas4 modulation following duloxetine treatment in SERT$^{-/-}$ rats, we also show that the impairment of the GABAergic system in the hippocampus and in the prefrontal

cortex of SERT$^{-/-}$ rats can 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.

In summary, since depression vulnerability can be associated with impaired neuronal plasticity (McClung and Nestler, 2008), we suggest that the reduced expression of BDNF in animals carrying a deletion of the SERT gene may contribute to their pathologic phenotype. This impairment originates early in development and is associated with reduction of transcription factors and GABAergic markers, suggesting that these defects may contribute to behavioral phenotype associated with SERT$^{-/-}$, and in particular those that are relevant for anxiety and depression. Moreover these animals maintain the ability to show neuroplastic changes in response to antidepressant drugs, possibly via the blockade of the noradrenaline transporter. The molecular characterization of the SERT mutant rat may prove useful to elucidate the pathophysiology of the depressive disorder and may eventually lead to the development of new effective treatments.

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