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Study of the role of the serotoninergic system in the brain functionality and in the vulnerability to psychopathologies: a genetic approach

BIO/14

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Abstract

Psychiatric pathologies are the second leading cause of disability worldwide with about 10% of the general population that manifest at least one symptom during their lifetime. This, together with the only partial effectiveness of the treatments adopted to date, make these illnesses an important burden for the society as well as for families and patients.

The first theory elaborated to explain the onset of psychiatric pathologies is the monoaminergic hypothesis that postulated how low levels of monoamines such as serotonin in the brain could be at the basis of depressive episodes and of other mood disorders. In line, human studies highlighted how alterations in different genes coding for different players involved in the serotoninergic system can be related to an increased susceptibility for psychopathologies.

Despite this linkage seems striking, different aspects of these pathologies cannot be explained with a direct causal relationship, making this hypothesis part of a bigger picture that also involves the environmental factors as well as other mechanisms.

On these bases, understanding the complexity of the systems that are modulated by serotoninergic alterations could give novel insights in understanding these such heterogeneous diseases.

To better clarify this aspect, during my PhD I took advantage of different genetically modified animal models characterized by alterations in the serotoninergic system. In particular, we evaluated the effect of low functionality of the serotonin transporter (SERT) on brain functions and we confirmed the pathological-like behavior of $SERT^{-/-}$ rats, characterized by a massive presence of serotonin in the extracellular compartments of the whole body. Moreover, we perform molecular analyses that confirmed alterations in different systems such as the neuroplastic mechanisms, the GABAergic system, and the dendritic spine functionality. Furthermore, we evaluated if a positive environment could ameliorate the behavior and the molecular abnormalities of $SERT^{-/-}$ rats finding a restorative effect of one month of enriched environment both on the anhedonic and anxiety-like behavior and on the molecular abnormalities.

Moreover, by altering the expression of the isoforms 1 or 2 of the tryptophan hydroxylase (TPH) enzyme, responsible for the production of serotonin in the periphery or in the brain respectively, we took into consideration $TPH1^{-/-}$ and $TPH2^{-/-}$ rats. More specifically, we worked on $TPH2^{-/-}$ rats, which do not have serotonin in the central nervous system and are characterized by an aggressive behavior and low anxiety levels at adulthood. Interestingly, we

found that the lack of brain-serotonin was counteracted by an upregulation in the neurotrophin Brain-derived neurotrophic factor (Bdnf) expression. Moreover, we found that this compensatory mechanism was present also at early adolescence, while at post-natal day 10, when the blood-brain-barrier is not completely formed and we cannot distinguish between central and peripheral serotonin, Bdnf expression was lowered. Interestingly, this increased neuroplasticity at adulthood was not enough to cope with an acute stress such as one hour of restraint stress. Indeed, while wild-type rats showed an activation of the Bdnf machinery and of the transcription of the immediate early genes, this effect was reduced in $TPH2^{-/-}$ rats. Accordingly, we found that the hypothalamic pituitary adrenal (HPA) axis and specifically its genomic pathway activation was much more consistent in $TPH2^{+/+}$ than in $TPH2^{-/-}$ rats.

Interestingly, we observed similar results also in $TPH1^{-/-}$ rats that showed a dramatic reduction in serotonin levels in different peripheral organs. Indeed, the lack of peripheral serotonin was able to reduce the anxiety-like behavior in these rats suggesting that also the peripheral pool of this molecule can alter brain functionality. Moreover, this positive effect on their behavior was sustained, at central level, by an increased expression of *Bdnf*. Nevertheless, also in this case, we found deficits in the reaction to the acute stress. Indeed, we found that the increased transcription of *Bdnf* and of the immediate early gene *Arc* happened specifically in wild-type while not in $TPH1^{-/-}$ rats. Similarly, the upregulation in the glucocorticorticoid responsive gene transcription was lower in a condition of absence of peripheral serotonin.

All the molecular analyses were conducted in the prefrontal cortex, a brain region highly innervated by serotoninergic fibers and involved in environmental changes response and in behavioral adaptations.

These results highlighted how these genetically modified animal models can be useful to study the role of serotonin in the modulation of brain functionality and the mechanisms involved in this process. Moreover, these findings supported the serotonin involvement in brain development and functionality. Ultimately, the data collected in this thesis give novel insights on the brain pathways affected by serotonin and on the role of this system in shaping the reaction to environmental stimuli in the field of psychiatric disorders.

Riassunto

Le patologie psichiatriche rappresentano la seconda causa di disabilità nel mondo e circa il 10% della popolazione ha manifestato almeno una volta nel corso della sua vita uno dei sintomi. Questo quadro è inoltre peggiorato dal fatto che i trattamenti ad oggi utilizzati sono solo parzialmente in grado di generare una piena remissione del paziente. Queste malattie rappresentano quindi un gravoso onere non solo per la società, ma anche per le famiglie oltre che per i pazienti stessi.

La prima ipotesi elaborata per spiegare la causa delle psicopatologie è stata l'ipotesi monoaminergica che sostiene come bassi livelli di monoammine come, per esempio, la serotonina possano essere alla base dell'insorgenza di episodi depressivi e di altri disordini dell'umore. A supporto di questa ipotesi, è stato dimostrato come alterazioni genetiche su diverse sequenze di DNA che codificano per componenti del sistema serotoninergico generino una aumentata suscettibilità allo sviluppo di patologie psichiatriche.

Sebbene questo legame possa sembrare estremamente chiaro, diversi aspetti di queste malattie non sono spiegabili con questa teoria con una diretta concausa e questo rende indispensabile pensare a questa ipotesi in un contesto più ampio che comprende anche fattori ambientali oltre che altri meccanismi.

Su queste basi, comprendere meglio la complessità dei sistemi che possono essere modulati da alterazioni serotoninergiche potrebbe portare a nuove conoscenze per una maggiore comprensione di questa patologie caratterizzate da una estrema eterogeneità.

Al fine di chiarire parte di questi aspetti, durante il mio dottorato di ricerca, abbiamo preso in considerazione diversi modelli animali geneticamente modificati e caratterizzati da alterazioni serotoninergiche. In particolare, abbiamo valutato come l'assenza del trasportatore della serotonina (SERT) possa avere un impatto sulle funzionalità cerebrali e abbiamo confermato il fenotipo patologico dei ratti $SERT^{-/-}$ che presentano elevati livelli di serotonina extracellulare in tutto il corpo. Inoltre, conducendo diverse analisi molecolari, abbiamo confermato alterazioni nei meccanismi di neuroplasticità, nel sistema GABAergico e nell'integrità delle spine dendritiche. Abbiamo quindi valutato se l'esposizione a un ambiente positivo potesse migliorare il quadro patologico dei ratti $SERT^{-/-}$ e abbiamo osservato un miglioramento sia dei comportamenti anedonico e ansioso che delle alterazioni molecolari dopo esposizione a un mese di ambiente arricchito.

Inoltre, alterando l'espressione delle isoforme 1 o 2 dell'enzima triptofano idrossilasi (TPH), responsabile della produzione della serotonina rispettivamente alla periferia e nel cervello,

abbiamo lavorato sui ratti TPH1$^{-/-}$ and TPH2$^{-/-}$. Nello specifico, abbiamo preso in considerazione i ratti TPH2$^{-/-}$, che non presentano serotonina nel sistema nervoso centrale e che mostrano un fenotipo aggressivo oltre che ridotti livelli di ansia durante la vita adulta. Abbiamo osservato come l'assenza di serotonina centrale fosse compensata da un aumento dei livelli di espressione della neurotrofina *Brain-derived neurotrophic factor* (Bdnf). Questo meccanismo compensatorio inoltre era presente a partire dalle prime fasi dell'adolescenza mentre, al giorno post-natale 10, quando la barriera emato-encefalica non è ancora completamente formata e non è possibile distinguere tra serotonina centrale e periferica, l'espressione di *Bdnf* risultava ridotta.

Tuttavia, abbiamo evidenziato come l'aumentata neuroplasticità non è sufficiente per permettere una adeguata risposta ad uno stimolo stressante come un'ora di *restraint* stress. Infatti, mentre gli animali wild-type hanno mostrato un aumento dell'espressione di *Bdnf* e degli *immediate early genes*, questo effetto era ridotto nei ratti TPH2$^{-/-}$. Allo stesso modo, abbiamo osservato come l'asse ipotalamo-ipofisi surrene e più nello specifico l'attivazione della sua via genomica era molto più consistente nei ratti TPH2$^{+/+}$ che in quelli TPH2$^{-/-}$.

Abbiamo infine osservato risultati simili anche per i ratti TPH1$^{-/-}$ che mostrano una drammatica riduzione dei livelli di serotonina periferica. Infatti, la mancanza della molecola nei distretti periferici è risultata in grado di ridurre i livelli di ansia di questi animali suggerendo la capacità dei livelli periferici della molecola di modulare le funzionalità centrali. Inoltre, questo effetto positivo a livello comportamentale è supportato da un aumento dei livelli di Bdnf nella corteccia prefrontale. Tuttavia, anche in questo caso abbiamo osservato deficit nella reazione dell'organismo a uno stress acuto. Abbiamo infatti osservato come l'espressione di *Bdnf* e dell'*immediate early gene Arc* era aumentata solamente negli animali wild-type e non nei ratti TPH1$^{-/-}$. Similmente, l'aumento dell'espressione dei geni responsivi ai glucocorticoidi dopo lo stress era ridotto in assenza di serotonina periferica.

Tutte le analisi molecolari sono state condotte nella corteccia prefrontale, una regione altamente innervata da fibre serotoninergiche e coinvolta nella risposta ai cambiamenti ambientali e nelle risposte comportamentali.

In conclusione, questi risultati dimostrano come questi modelli animali transgenici siano utili per studiare il ruolo della serotonina nella modulazione della funzionalità cerebrale e i meccanismi coinvolti in questo processo. Inoltre, questi dati supportano il coinvolgimento della serotonina sia nello sviluppo cerebrale che nella sua funzionalità. Infine, i dati raccolti

rappresentano nuove informazioni sui sistemi alterati dalla serotonina e sul ruolo del sistema serotoninergico nella modulazione della risposta a eventi ambientali nel contesto delle patologie psichiatriche.

1. Introduction

1.1. Serotonin

Serotonin, also known as 5-hydroxytryptamine (5-HT), is widely distributed in animals and plants (Srinivasan et al., 2008; Kang et al., 2009; Curran and Chalasani, 2012). The first insights related to this molecule date back to the 40s when a group of researchers isolated a vasoconstrictor substance which was named serotonin (Rapport et al., 1948a, 1948b; Rapport, 1949). In the meantime, in Italy, the group of Vittorio Erspamer at the University of Pavia extracted an amine-containing vasoconstrictor from rabbit gut mucosa, and that they called it enteramine (Renda, 2000). A few years later, they demonstrated that enteramine and serotonin were the same molecule shown in Figure 1 (Erspamer and Asero, 1952).

Interestingly, in 1953, serotonin have been identified also in mammals' brain (Twarog and Page, 1953) and in 1964 Annika Dahlström and Kjell Fuxe, two Swedish scientists, determined the brain regions in which it was distributed (Dahlström and Fuxe, 1964).

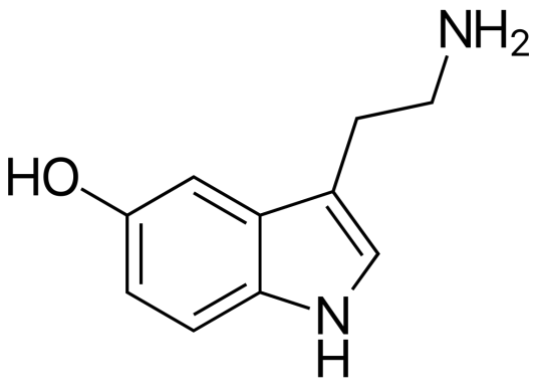

Figure 1: serotonin structure

1.1.1. Serotonin synthesis and transmission

Serotonin biosynthesis is a two-step process: the first step happens through the action of the rate-limiting enzyme named Tryptophan hydroxylase (TPH) that catalyzes the hydroxylation of the essential amino acid tryptophan to 5-hydroxytryptophan (5-HTP). Then, 5-HTP is decarboxylated to serotonin via the action of the aromatic amino acid decarboxylase (AADC) enzyme (Walther et al., 2003) (Figure 2).

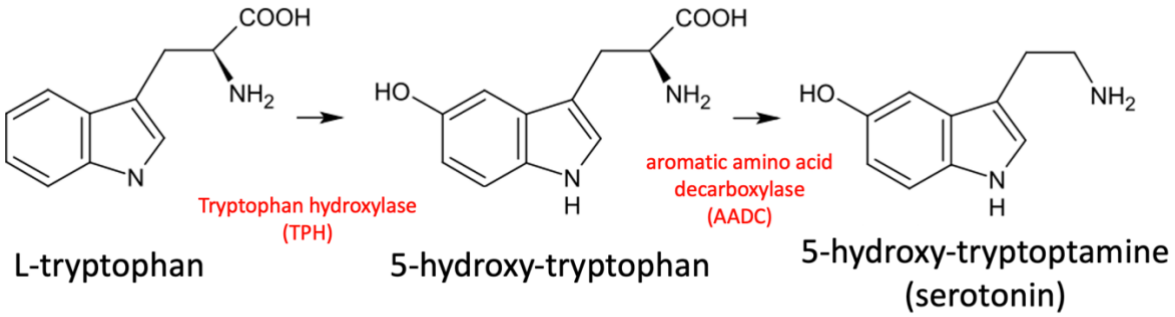

Figure 2: serotonin synthesis

The TPH enzyme is a monooxygenase with iron (Fe^{2+})- and tetrahydrobiopterin (BH4)-dependent action. This enzyme belongs to the aromatic amino acid hydroxylases enzyme superfamily, and it is characterized by three functional domains: a N-terminal regulatory domain, a catalytic domain, and a C-terminal oligomerization domain (Fitzpatrick, 1999; Veenstra-VanderWeele et al., 2000; Mosienko et al., 2015). Only in 2003, it has been discovered the presence of two different isoforms of the TPH enzyme: the TPH1, acting in the periphery and mainly distributed in the enterochromaffin cells, in the thymus and in the pineal gland, and the TPH2 enzyme responsible for the production of serotonin in the brain (Walther and Bader, 2003; Walther et al., 2003; Zhang et al., 2004; Zill et al., 2004; McKinney et al., 2005). Interestingly, in humans, the two isoforms share about 71% of the amino-acidic sequence and they have a very similar tridimensional structure, giving to these two enzymes the same functionality but a different distribution, solubility, and affinity for the substrate (Walther et al., 2003; McKinney et al., 2005).

Considered the different distribution of these two isoforms and the inability of the serotonin to pass through the blood-brain barrier, we can identify two distinct pools of the molecule: one at the periphery and the other in the central nervous system (CNS) (Berger et al., 2009).

As shown in Figure 3, once it is produced, serotonin is stored into vesicles via the action of the vesicular monoamine transporter type 2 (VMAT2) (De-Miguel et al., 2015) and, after different stimuli, it is released through a calcium-dependent mechanism to activate its receptor (Filip and Bader, 2009). Finally, it is re-uptaken thanks to the action of the serotonin transporter (SERT) that controls the strength and the duration of the serotoninergic activity (Canli and Lesch, 2007; Olivier et al., 2009). Indeed, the transporter SERT is a member of the Na^+/Cl^--dependent solute carrier 6 (SLC6) family characterized by 12 α-helical transmembrane domains with 6 extracellular and 5 cytoplasmic loops (Kristensen et al., 2011; Bröer and Gether, 2012) and it is able to remove the serotonin from the extracellular compartments in a 1:1 stoichiometry (Rudnick and Sandtner, 2019).

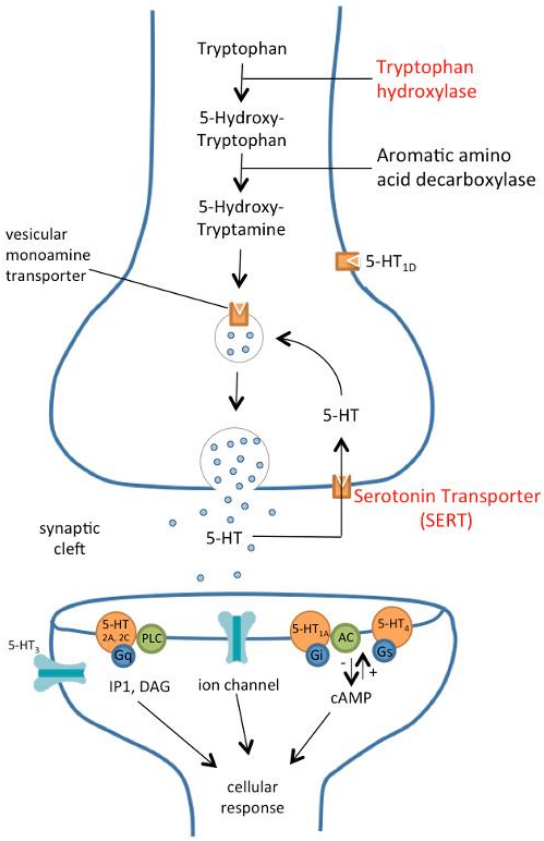

Figure 3: serotoninergic transmission

1.1.2. Serotonin functions

As mentioned, serotonin plays its role both in the central nervous system (CNS), acting as neurotransmitter, and at the periphery where it acts as an autacoid (De-Miguel et al., 2015; Hainer et al., 2015).

Central serotonin, synthesized in the raphe nuclei, is about 5% of the total body amount of the molecule in the body. However, in the brain, it is responsible for the control of a wide range of physiological and behavioral processes. Indeed, thanks to different projections that originate from the raphe nuclei (Figure 4), serotonin can control sleep, food assumption, sexual behavior and learning and memory processes, but also the mood by inducing a sense of happiness and cheerfulness. Moreover, it is involved in the control of aggressive and impulsive behavior as well as pain perception and locomotor activity (Lucki, 1998; Veenstra-VanderWeele et al., 2000; Gross and Hen, 2004; Miczek et al., 2007; Lesch et al., 2012; Alenina and Klempin, 2015; Pourhamzeh et al., 2021).

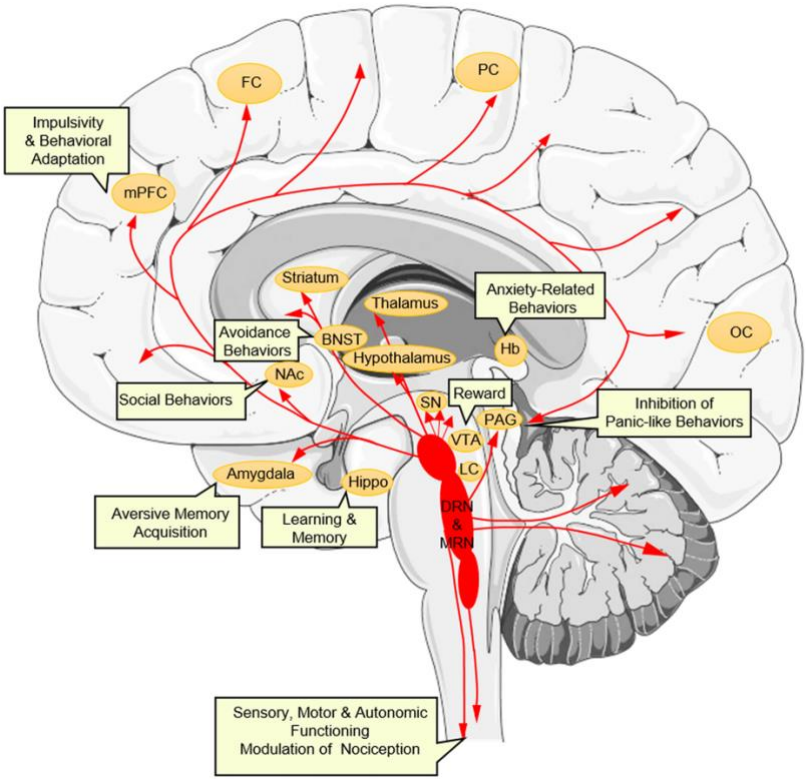

Figure 4: serotonin projection originated from the raphe nuclei (adapted from Pourhamzeh et al., 2021).

Interestingly, during the first stages of life, serotonin plays its role also in the development acting as trophic factor: indeed, it modulates cell proliferation, migration but also cell survival and synaptogenesis (Buznikov et al., 2001; Whitaker-Azmitia, 2001; Gaspar et al., 2003). In line, serotonergic neurons are among the first to be formed and they can release serotonin before reaching complete maturation. Also, serotonin is present in the brain before the complete formation of the synapses and in the perinatal life many neurons show a partial serotonergic-like phenotype and they can store and release serotonin without synthesizing it (Whitaker-Azmitia, 2001; Gross et al., 2002). This process of transient expression of the SERT and of the VMAT2 could also be useful to prevent an excessive amount of serotonin in the brain when the blood-brain barrier is not completely formed and the peripheral serotonin as well as mother-derived serotonin can easily reach the CNS (Trowbridge et al., 2011).

The other 95% of body serotonin is located at the periphery where it controls a wide range of physiological functions (Figure 5). The vast majority is synthesized in the gut where it promotes motility and fluid secretion (Gershon and Tack, 2007). Gut-derived serotonin is also responsible for smooth muscle contraction and stomach nerve stimulation evoking nausea (Jenkins et al., 2016). Other than acting in the gastrointestinal tract, peripheral serotonin is also released in the bloodstream where it is stored into the platelets allowing blood coagulation and where it acts as vasoconstrictor controlling blood pressure levels (Yildiz et al., 1998).

Interestingly, peripheral serotonin is also involved in the metabolic homeostasis control since it contributes to insulin secretion, gluconeogenesis, and lipolysis (Paulmann et al., 2009; Kim et al., 2010; El-Merahbi et al., 2015). Different evidence shows how 5-HT has a role in inflammation and immunity with different immune cells that appear to be able to synthesize, transport and store serotonin (Jackson et al., 1988; Kushnir-Sukhov et al., 2007; Kritas et al., 2014; Chen et al., 2015). Finally, there's a strong interplay between serotonin and microbiome (Reigstad et al., 2015; Yano et al., 2015).

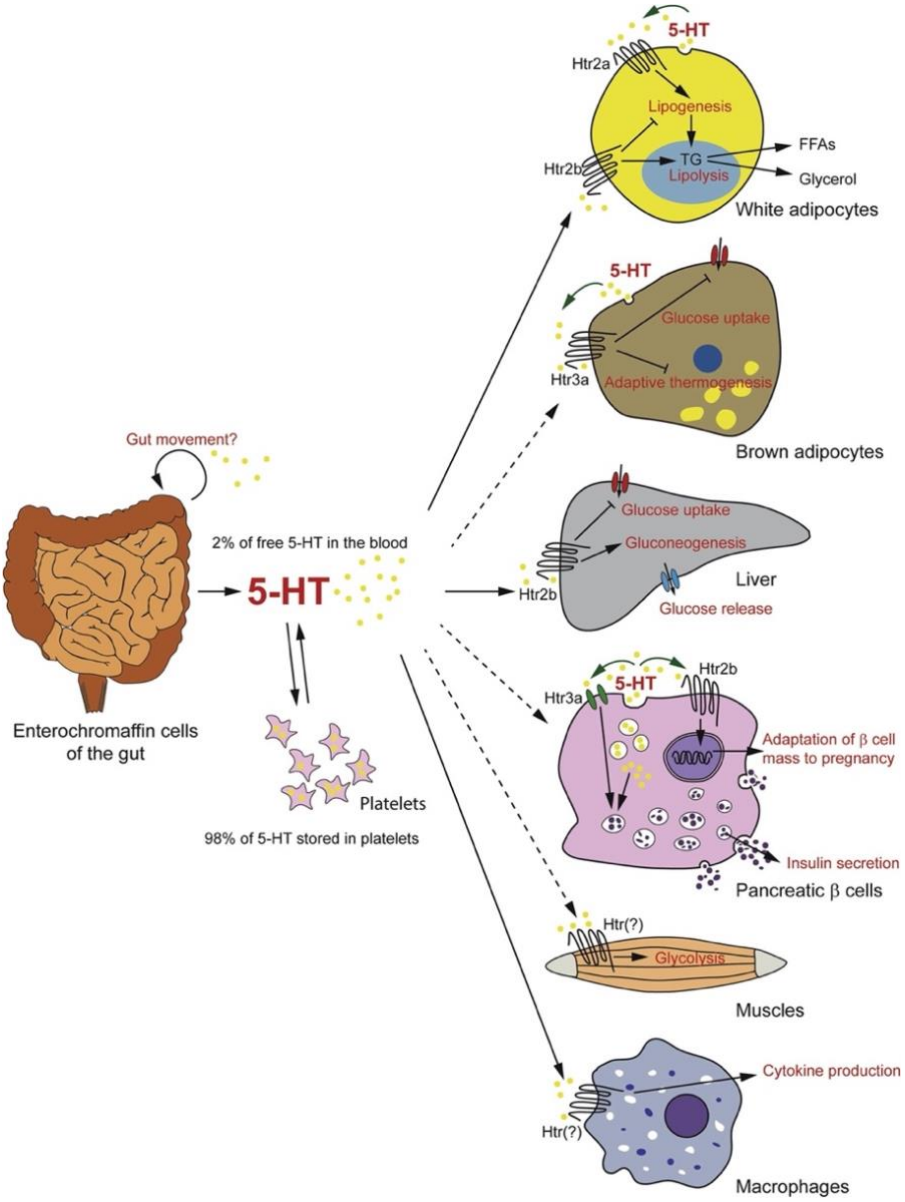

Figure 5: different roles of peripheral serotonin (adapted from (El-Merahbi et al., 2015).

1.1.3. Serotonergic alterations and psychopathologies

One of the first hypotheses to explain psychiatric pathologies' origin was the monoamine neurotransmitter depletion theory that postulated how decreased levels of monoamines such as serotonin in the CNS could be at the basis of depressive episodes (Krishnan and Nestler, 2008).

This hypothesis derives from the accidental discovery that drugs that increase the monoaminergic tone were able to generate an antidepressant effect. Based on this evidence, many compounds that improve the monoaminergic transmission have been developed and widely administered to people suffering from psychopathologies with positive results. However, only one third of patients have a complete remission and, even when the treatment is successful, the antidepressant effect is delayed and occurs only after several weeks (Trivedi et al., 2006). This places the monoaminergic hypothesis in a broader picture that includes also other systems. Accordingly, the implication of other mechanisms better reflects the extreme heterogeneity of these illnesses.

Still, the role of serotonin in the etiopathogenesis and in the development of psychiatric illnesses is central and widely demonstrated even if the mechanisms are not completely clarified yet.

1.1.3.1. The serotonin transporter promoter region polymorphism

As mentioned, SERT is the most important regulator of serotonergic function controlling its concentration in the extracellular compartments and thus its action.

The human gene coding for the SERT, the *SLC6A4*, is located in the chromosome 17q11.2 and is composed of 15 exons spanning ~40 kb and coding for 630 amino acids sequence. The transcription of this gene leads to multiple mRNA species due to alternative promoters, different splicing sites and the variability of the untranslated regions (Murphy et al., 2008). Among them, the most studied is for sure the polymorphism of the promoter region of the serotonin transporter (5-HTTLPR) which modulate the transcription of the gene. As shown in Figure 6, deletions or insertions of the repetitive sequence of the promoter lead to short allele with 14-repeats or to the long allele composed of 16-repeats of the sequence. Notably, 17 up to 22 repeat alleles (extra-long allele) have been identified as well (Goldman et al., 2010). Moreover, a single nucleotide polymorphism (SNP) close to the *SLC6A4* gene leads to two different forms of the L-allele (L-A and L-G) as well as of the S-allele (S-A and S-G) and all these

modifications significantly affect the transcription level of the SERT. In particular, the short allele carriers show about 60-70% lower SERT mRNA levels compared to people with long-allele (Murphy et al., 2008; Wankerl et al., 2014). Furthermore, L-G variants produce transcription levels similar to the short-allele (Hu et al., 2006).

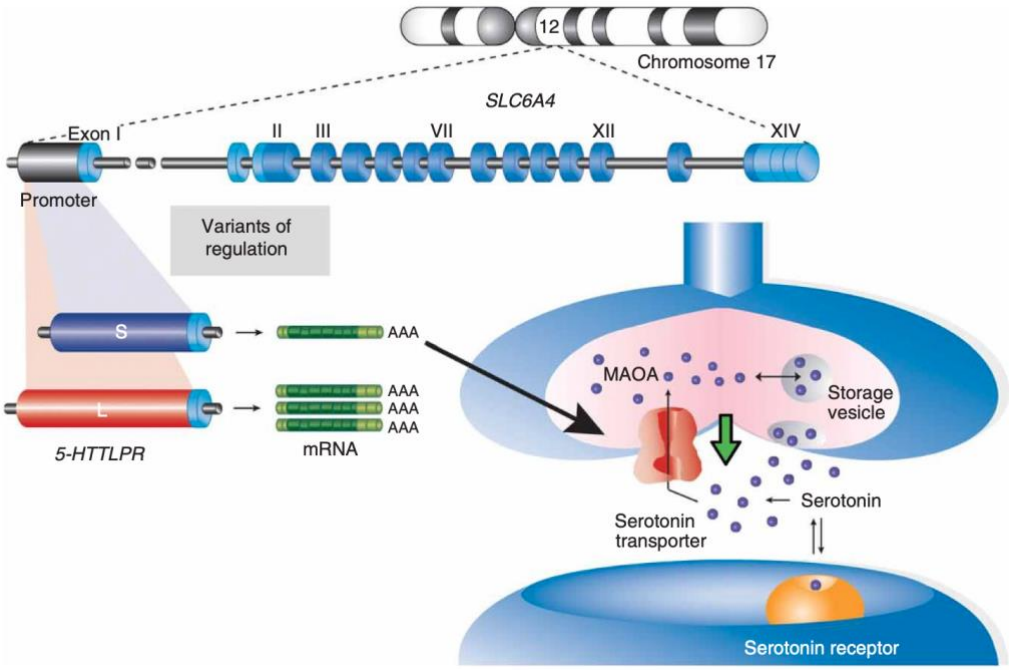

Figure 6: allelic variation of serotonin transporter. The short (S) 5-HTTLPR allelic variant is colored in purple and produces less amount of mRNA while the long allelic variant (in red) results in larger transcripts rate (adapted from Canli and Lesch, 2007).

Interestingly, in 1996 it has been postulated that low expressing SERT individuals characterized by the presence of one or two copies of the short allele more likely developed neuroticism (Lesch et al., 1996). Accordingly, following studies supported these first insights suggesting a relationship between short allele and depression, anxiety and mood disorders related to emotions regulation (Mazzanti et al., 1998; Greenberg et al., 2000; Caspi et al., 2003). In particular, 5-HTTLPR short variant has been associated with an inability to cope with negative environmental stimuli both during the development and at adulthood resulting in the manifestation of different symptoms of depression while other studies showed results against this relationship (Sen et al., 2004; Munafò et al., 2009; Risch et al., 2009; Terracciano et al., 2009; Karg et al., 2011; McGuffin et al., 2011; Sharpley et al., 2014; Vrijsen et al., 2015). Hence, the role of the SERT polymorphisms in the field of psychiatric disorders remains controversial and to be better explored.

1.1.3.2. Tryptophane hydroxylase genetic polymorphisms

Other than 5-HTTLPR variants, also the genes coding for the TPH enzyme present different SNPs that could be related to psychopathologies. Moreover, low levels of serotonin have been found in people suffering from mood disorders (Andrews et al., 2015).

The genes encoding for TPH1 and TPH2 enzymes are located, in humans, in the chromosomes 11p15.1 and 12q21.1 respectively. These two genes encode for two highly homologous aminoacidic sequences forming a very similar structure of the proteins (McKinney et al., 2005) that differ for the molecular weight, kinetic properties, and phosphorylation site other than their localization in the brain or in the periphery (Winge et al., 2007, 2008; Cichon et al., 2008; McKinney et al., 2008, 2009).

As well revised in Ottenhof et al., 2018, the TPH2 gene presents different human variants in the coding and in the non-coding region of the gene that showed a reduced transcription of the enzyme (Scheuch et al., 2007; Chen et al., 2008) and have been associated to different pathological conditions such as attention-deficit/hyperactivity disorder (ADHD), (Sheehan et al., 2005; Walitza et al., 2005; Brookes et al., 2006), obsessive-compulsive disorder (Mössner et al., 2006); depression (Zill et al., 2004; Zhang et al., 2005; Gao et al., 2012; Kulikov and Popova, 2015; Yang et al., 2019), bipolar disorder (Gao et al., 2016) but also to impairments in the regulation of emotional processing and behavior (Brown et al., 2005; Canli et al., 2005; Herrmann et al., 2006). However, other studies did not find any association between TPH2 polymorphisms and psychopathologies or suicidal behavior (Johansson et al., 2010; González-Castro et al., 2014; Uka et al., 2019).

Interestingly, despite the localization of the TPH1 isoform at peripheral level, SNPs in this gene have been associated not only with irritable bowel disease (Katsumata et al., 2018) but also with brain-related pathologies including depression (Viikki et al., 2010; Wigner et al., 2018), post-traumatic stress disorders (PTSD) (Goenjian et al., 2012), alcohol abuse (Chen et al., 2012). Also, in the case of this peripheral isoform, other studies failed to prove the relationship between SNPs and mood disorders (Johansson et al., 2010; Khabour et al., 2013).

1.2. Multifactorial etiology for psychopathologies

As explained above, different players of the serotonergic system show a wide range of genetic alterations that have been linked to psychopathologies and mood disorders. However, we could not identify a direct causal relationship between the presence of a genetic alteration and the onset of the symptoms. Hence, the presence of genetic polymorphisms is only a risk factor rather than the cause of psychopathologies. In line, while some genome-wide association studies demonstrated a direct association even if with small effect size, others failed to prove this connection without any reasonable doubt (Wellcome Trust Case Control Consortium, 2007; Ferreira et al., 2008; O'Donovan et al., 2008; Sullivan et al., 2009; Muglia et al., 2010).

The non-linear association between these genetic modifications and the pathological state of people who carry them is mainly due to the environment in which individuals are surrounded. Consistently, it has been demonstrated that psychopathologies are characterized by a multifactorial etiology with the genetic make-up that is as important as the environment. Thus, the presence of serotonergic mutations plays its role as a risk factor and its effect is unmasked only in negative contexts (Duncan et al., 2014).

Interestingly, it has been recently postulated that, in people with genetic alterations in the serotonergic system, negative situations are able to induce a worst outcome while positive situations can produce better results making these individuals more sensitive to external stimuli. Thus, this differential susceptibility theory suggests that people carrying those candidate genes linked to psychopathologies are both disproportionally vulnerable to adversities as well as they have great benefits deriving from positive and enriched environments. Hence, these genes could be considered plasticity genes instead of vulnerability genes (Belsky et al., 2009).

1.2.1. Enriched environment as positive stimulus

While the effect of negative situations such as stress exposure, childhood trauma or abuses are well described in literature, how a positive environment can shape brain function and improve the individual well-being is for sure less explored especially in clinical studies.
However, recent preclinical studies tried to mimic a positive environment both by improving the housing or social conditions but also by increasing the motor activity in order to better understand how it can produce its positive influence. In this context, an enriched environment could be considered as a combination of both inanimate and social supplementations during the lifespan (Rosenzweig et al., 1978). Interestingly, it has been demonstrated how this housing condition can ameliorate or slow the progression of different brain-related pathologies including Parkinson's disease (Bezard et al., 2003; Kleim et al., 2003), Alzheimer disease (Adlard et al., 2005), drug addiction (Thiriet et al., 2005; Solinas et al., 2009) and anxiety and depressive-like traits (Sparling et al., 2020).

1.2.2. Stress as negative stimulus

Among the negative stimuli that an organism can be exposed to, the stress is for sure the most investigated in the field of psychopathologies. Despite the negative acceptation of stress, it actually can be good stress, tolerable stress or toxic stress depending on the time and on the length of the exposure (Shonkoff et al., 2009). Particularly, in healthy subjects, stress induces many changes that altogether result in an inverted U shape dose-response relationship as represented in Figure 7. Indeed, short periods of stress in terms of minutes to hours have a positive effect enhancing brain functions and activating a wide range of positive systems such as the transcription of the brain-derived neurotrophic factor (Bdnf), the release of corticotrophin releasing factor (CRF) and the activation of the endocannabinoids system that in turn allow the necessary brain remodeling (Chen et al., 2006; Govindarajan et al., 2006; Hill and McEwen, 2010; Lakshminarasimhan and Chattarji, 2012; Brivio et al., 2020b). On the contrary, long periods of stress in terms of months to years are detrimental leading to maladaptive responses and to the suppression of these systems activation (McEwen et al., 2015; Calabrese et al., 2016, 2017, 2020; Brivio et al., 2020a). Accordingly, acute stress exposure is able to improve different functions of the organism making the individual able to cope with the negative situation but also improving the behavioral functions. On the contrary, chronic stress can produce the manifestation of maladaptive responses that inhibit the

organism's functionality and promote the manifestation of different pathologies including mood disorders (McEwen et al., 2015; Russell and Lightman, 2019).

However, in those susceptible subjects also brief stressful episodes could unmask some latent molecular alterations that in turn can induce a negative response and make the individual more susceptible to develop psychopathologies.

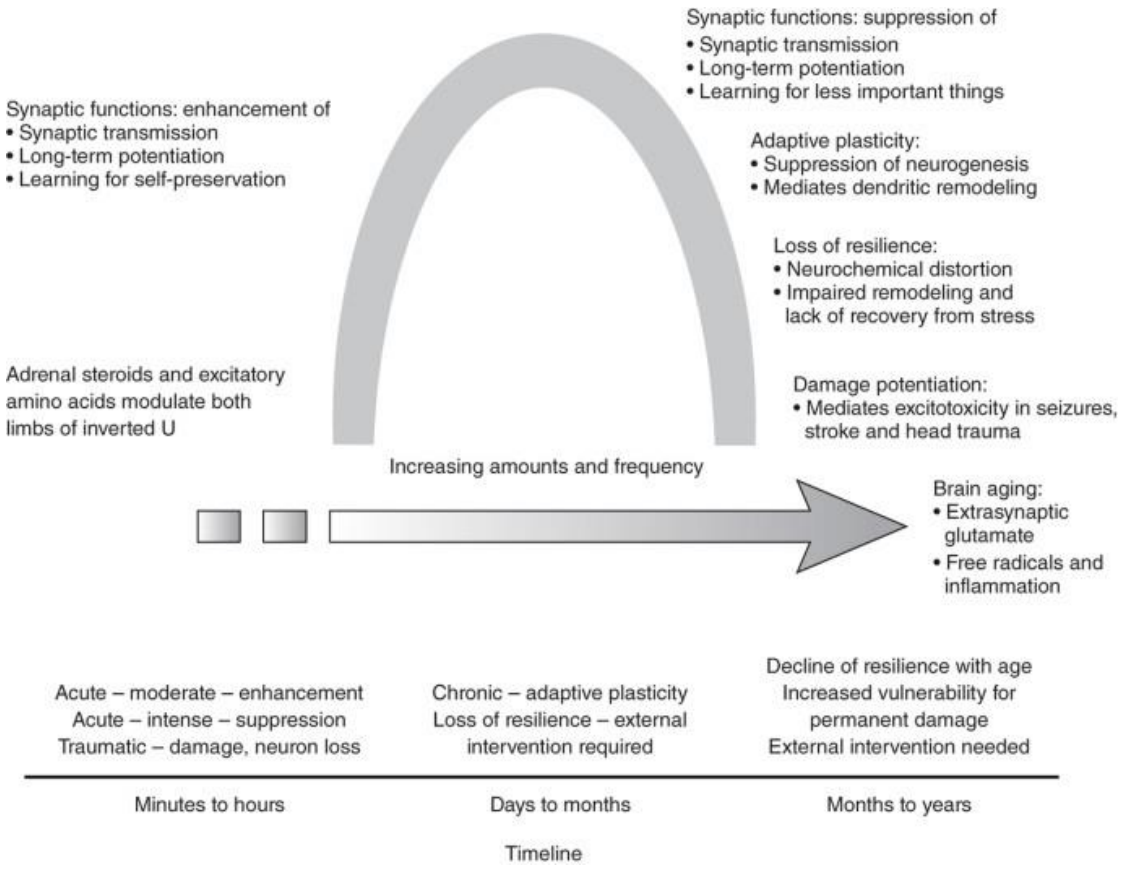

Figure 7: effect of acute vs chronic stress exposure represented as an inverted U shape dose-response curve (adapted from McEwen et al., 2015).

2. Aim

The world perception is extremely subjective, and everyone can respond differently to many environmental stimuli, both positive and negative. This is particularly true for psychopathologies that are characterized by a multifactorial etiology with the genetic vulnerability that becomes manifest specifically when the subject has to face negative contexts (Caspi et al., 2003; Duncan et al., 2014). Among the genetic factors, the ones related to the serotonergic system are for sure the most studied.

Interestingly, only 5% of the whole body serotonin is localized in the brain and it controls a wide range of physiological processes including sleep, mood, sexual behavior and also the proper development of brain circuits during the early stages of life (Lucki, 1998; Veenstra-VanderWeele et al., 2000; Buznikov et al., 2001; Whitaker-Azmitia, 2001; Gaspar et al., 2003; Gross and Hen, 2004; Miczek et al., 2007; Lesch et al., 2012; Alenina and Klempin, 2015; Pourhamzeh et al., 2021). The other 95% instead is distributed in the periphery where it regulates gut motility and digestion as well as metabolic functions (Gershon and Tack, 2007; Paulmann et al., 2009; Kim et al., 2010; El-Merahbi et al., 2015; Jenkins et al., 2016).

On these bases, it's not surprising how perturbations of this system have been linked to the development of pathologies of the gastrointestinal tract such as irritable bowel disease (Katsumata et al., 2018) but also to the manifestation of different psychopathologies such as depression, schizophrenia, anxiety, and other mood disorders (Krishnan and Nestler, 2008; Andrews et al., 2015).

In particular, it has been shown that the genes coding for the SERT, the TPH1 and TPH2 are characterized by the presence of different and quite diffuse polymorphisms that reduce the functionality of the transporter or of the enzymes thus increasing the probability to develop psychiatric illnesses (Lesch et al., 1996; Mazzanti et al., 1998; Greenberg et al., 2000; Caspi et al., 2003; Ottenhof et al., 2018). However, other studies failed to prove this direct relationship (Johansson et al., 2010; Sharpley et al., 2014). The inconsistency between these findings could be justified by differences in the social and in the emotional context of life of people carrying these gene mutations: a recent theory indeed states that serotonergic dysfunctions not only worsen the impact of a negative situation and the reaction to it but also amplifies the response to positive and enriched stimuli (Belsky et al., 2009; van der Doelen et al., 2014). This is also supported by the fact that these genetic alterations are maintained throughout the evolution: if they exerted a specific negative effect their diffusion would be reduced through natural selection (Wendland et al., 2006).

Finally, despite the evidence linking these genes to a greater sensitivity to the surroundings, the systems involved, and the mechanisms activated in positive or negative contexts are not fully clarified yet.

On these bases, the aim of this thesis is to evaluate the impact of a disrupted serotoninergic system on the organism's functionality by taking advantage of different transgenic animal models and by evaluating their conditions at basal level. Moreover, we evaluated their response to different environments both positive and negative focusing on the impact of these external stimuli both on the behavior and on the molecular abnormalities.

In particular, to mimic the polymorphism of the serotonin transporter, which is not present in rodents, we took advantage of the $SERT^{-/-}$ rats that showed a clear behavioral and molecular pathological phenotype (Olivier et al., 2008; Kiser et al., 2012). Hence, we evaluated if the exposure to one month of enriched environment could be beneficial and ameliorate or restore the pathological-like alterations of this animal model.

Moreover, to better understand how alterations in serotonin levels both in the brain and at the periphery could be a risk factor, we worked on $TPH1^{-/-}$ and $TPH2^{-/-}$ rats characterized by a complete absence of serotonin at the periphery or in the brain respectively (Kaplan et al., 2016). Seen poor and controversial results present in literature on the depressive-like behaviors of rodents lacking central or peripheral serotonin (Mosienko et al., 2012; Suidan et al., 2013; Kaplan et al., 2016; Peeters et al., 2019), here, we exposed $TPH1^{-/-}$ and $TPH2^{-/-}$ rats to an acute challenge in order to evaluate their stress responsiveness. Indeed, in a healthy subject, an acute stress positively activates the system allowing the organism to cope with it (Chen et al., 2006; Govindarajan et al., 2006; Hill and McEwen, 2010; Lakshminarasimhan and Chattarji, 2012; Brivio et al., 2020b). However, in an organism with alterations in vulnerability genes such as TPH1 and TPH2, this negative stimulus may unmask some molecular scars that could ultimately predispose to psychopathologies.

All in all, the purpose of this thesis is to better dissect how serotonergic alterations could modulate the organism by altering the behavior and the molecular functionality of $SERT^{-/-}$, $TPH1^{-/-}$ and $TPH2^{-/-}$ animal models at basal level. Moreover, we evaluated if disruptions of the serotoninergic system could shape the reaction to different positive or negative environments and ultimately act as a risk factor in the field of psychopathologies.

3. Material and methods

3.1. Animals and experimental paradigms

3.1.1. SERT$^{-/-}$

SERT$^{-/-}$ rats were obtained through N-ethyl-N-nitrosourea (ENU) mutagenesis, on a Wistar background as described by Smits and colleagues (Smits et al., 2004, 2006). SERT$^{-/-}$ and SERT$^{+/+}$ rats were reared at the Central Animal Laboratory of the University of Nijmegen Radboud UMC. From the birth, the animals were housed under standardized conditions involving a 12/12h light/dark cycle, at 22°C and around 80% of humidity with access ad libitum to food and water. After paired housing conditions from weaning, at adulthood, SERT$^{-/-}$ and SERT$^{+/+}$ male rats were assigned to either of the two experimental groups and housed in a normal or enriched cage in a random way. Normal environment involved paired housing in a cage sized 42.5x26.6x18.5cm enriched with a rat retreat, while enriched environment consisted of groups of 10 animals housed in cages sized 100x54.5x48cm with cage enrichment that included toys, tunnels, and nesting places of different colors and textures to promote exploration behavior. Both normal and enriched environment experimental groups were housed in the same room, and all the animals were handled once a week. From day 15 to day 24 of the housing procedure, we performed behavioral tests following the schedule represented in Figure 8. At the end of the 31st day, animals were decapitated to perform the molecular analyses. All experimental procedures were approved by the Central Committee on Animal Experiments (Centrale Commissie Dierproeven, CCD, The Hague, The Netherlands), limiting the number of animals used and minimizing animal suffering.

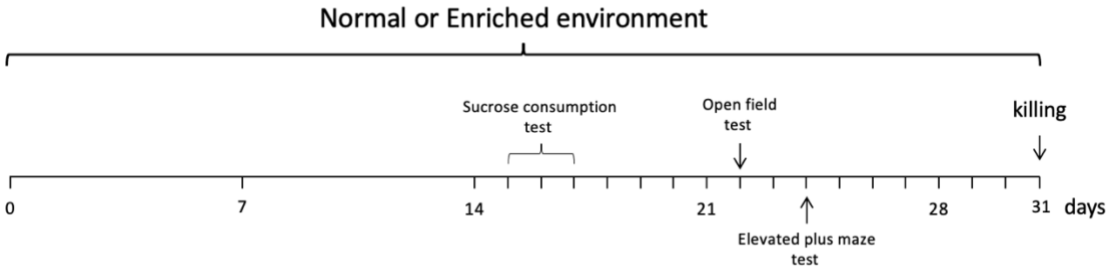

Figure 8: graphical representation of the experimental paradigm. SERT$^{+/+}$ and SERT$^{-/-}$ rats were exposed to one month of enriched environment. The sucrose consumption test was performed on day 15 to 17, the open field test was conducted on day 22 and the elevated plus maze test on day 25 after the start of the EE. On day 31, animals were sacrificed for the subsequent molecular analyses.

3.1.2. TPH2$^{-/-}$

TPH2$^{-/-}$ rats were obtained using the zinc-finger nucleases technique on a Dark Agouti background as described by Kaplan and colleagues (Kaplan et al., 2016). TPH2$^{-/-}$ and TPH2$^{+/+}$ rats were reared at the facility of Max-Delbrück Center for Molecular Medicine in Berlin. From the birth, the animals were housed under standardized conditions involving a 12/12h light/dark cycle, at 22°C and around 80% of humidity with access ad libitum to food and water. For the basal characterization, a cohort of TPH2$^{+/+}$ and TPH2$^{-/-}$ male and female rats were left undisturbed until adulthood (12–14 weeks of age) when they were decapitated for the subsequent molecular analyses. Another cohort of rats was sacrificed through decapitation at different time points during the development, specifically at post-natal day 1, 10 or 30 to perform the molecular analyses during the ontogenesis. Finally, a third cohort of adult male TPH2$^{+/+}$ and TPH2$^{-/-}$ rats were tested for stress responsiveness. In particular, TPH2$^{+/+}$ and TPH2$^{-/-}$ rats were exposed to acute restraint stress that consisted in placing the animals into an air-accessible apparatus for one hour. As represented in Figure 9, the size of the container was similar to the size of the animal which made the rats almost immobile in it. Part of the stressed animals was sacrificed right after the end of the stress while the others were placed back in their home cage for one hour and then sacrificed through decapitation. No stressed animals were left undisturbed in their home cage until the sacrifice.

All the procedures were approved by the ethical committee of the local government (LAGeSo, Berlin, Germany) limiting the number of animals used and minimizing animal suffering.

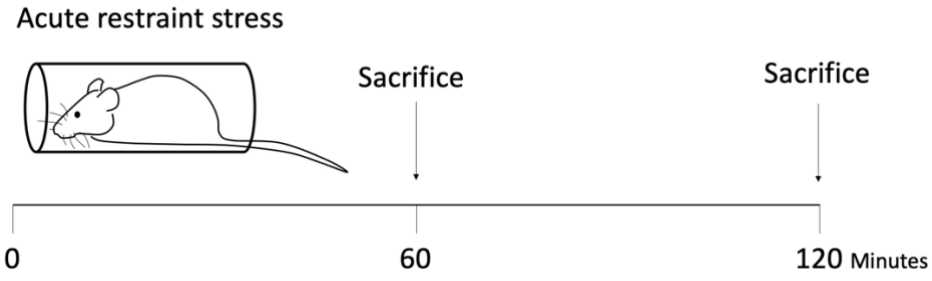

Figure 9: graphical representation of the experimental paradigm. After the acute restraint stress (1h), wild-type and knock-out rats were sacrificed right after the end of the stress or one hour later for the subsequent molecular analyses.

3.1.3. TPH1$^{-/-}$

TPH1$^{-/-}$ rats were obtained at the Gene Editing Rat Resource Center Of The Medical College Of Wisconsin by injecting a Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) targeting the Tph1 gene in Wistar Kyoto rats producing a deletion in the exon 4. The breeding was conducted at the Central Animal Laboratory of the University of Nijmegen Radboud UMC. From the birth, the animals were housed under standardized conditions involving a 12/12h light/dark cycle, at 22°C and around 80% of humidity with access ad libitum to food and water. At adulthood, TPH1$^{+/+}$ and TPH1$^{-/-}$ rats were tested for their anxiety level during the elevated plus maze test. Moreover, two weeks later, half of the animals were tested for their stress responsiveness by exposing them to one hour of acute restraint. The apparatus adopted had the same characteristics as the one used for TPH2$^{-/-}$ rats (Figure 9). Right after the stress, animals were anesthetized and sacrificed through decapitation. No stressed animals were left undisturbed in their home cage until the sacrifice.

All experimental procedures were approved by the Central Committee on Animal Experiments (Centrale Commissie Dierproeven, CCD, The Hague, The Netherlands–ethic), limiting the number of animals used and minimizing animal suffering.

3.2. Behavioral tests

3.2.1. Sucrose Consumption

Anhedonia, a core feature of depression, was investigated with the sucrose consumption test, and the anhedonic-like behavior refers to a reduction of the preference for the sucrose solution over plain water during the test (Willner et al., 1987). As pictured in Figure 10, for this procedure, $SERT^{-/-}$ and $SERT^{+/+}$ animals were single-housed for 4 h a day (for three days—two of habitation and one of the test), and they had free access to two bottles: one filled with water and one of 4% sucrose solution. Fluid consumption (g) was measured by weighing the bottles before and 4 h after the start of the test, and the measures were used to calculate sucrose preference (sucrose intake in ml divided by total intake × 100%).

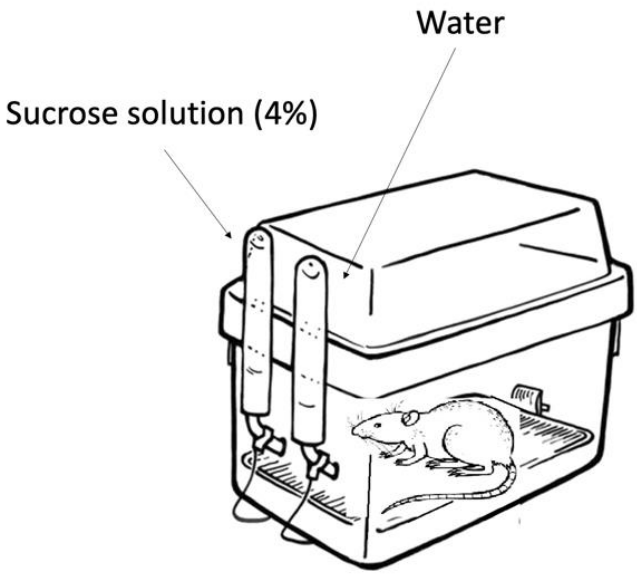

Figure 10: sucrose consumption test

3.2.2. Open Field

Taking advantage of the natural predisposition of rodents to explore new environments and spaces and to assess novelty-induced locomotor activity, we exposed the animals to the open field test by placing each rat in a squared arena (1 m × 1 m) and by letting them free to move and explore the novel environment for 5 min. As shown in Figure 11, $SERT^{-/-}$ and $SERT^{+/+}$ rats were placed in the center of the arena and were recorded. The distance moved, as well as the velocity, were measured using EthoVision XT (version 3.1., Noldus) software (Noldus, Wageningen, The Netherlands).

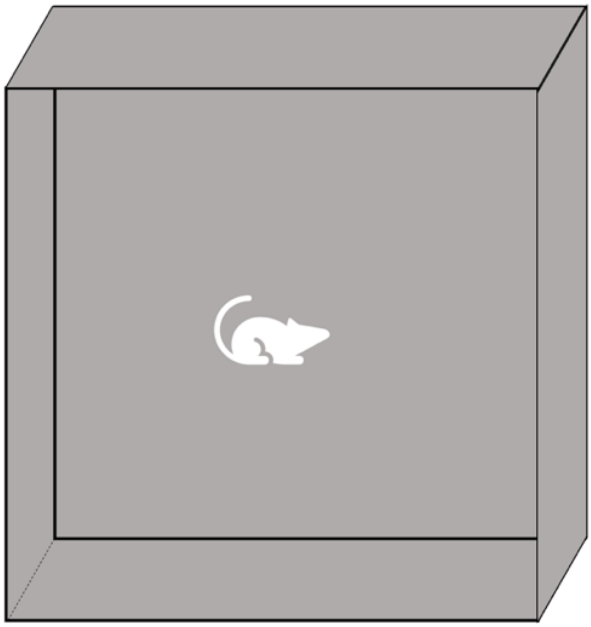

Figure 11: open field test

3.2.3. Elevated Plus-Maze (EPM)

Considering the innate fear of rodents for open and elevated spaces, we employed the EPM test to evaluate the anxiety-like behavior of the animals. In particular, a plus-shaped platform was located at 50 cm from the floor. The maze, represented in Figure 12, consisted of two closed arms (50 cm × 10 cm with 40 cm high walls) and two open arms of the same dimensions and without walls. The two equal arms were positioned opposite each other, and the four arms formed a central platform at their intersection. At the beginning of the test, each $SERT^{-/-}$ and $SERT^{+/+}$ or $TPH1^{+/+}$ and $TPH1^{-/-}$ rat was placed on the central platform looking at one of the open arms. The position and the movements of the animals were recorded for 5 min, and the time spent in the closed arms, in the center, and in the open arms of the arena were analyzed using EthoVision XT (version 3.1., Noldus) software.

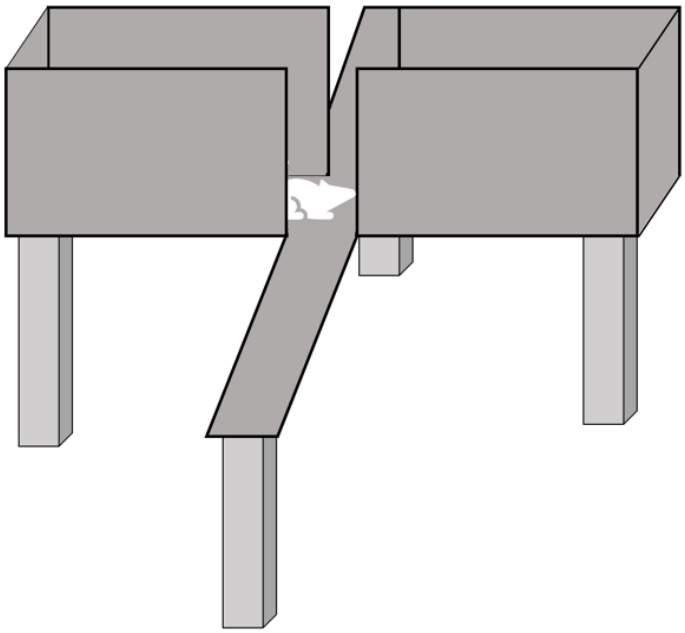

Figure 12: elevated plus maze test

3.3. Brain tissue collection

To perform the molecular analyses, the prefrontal cortex (PFC), or the frontal lobe for the developmental analyses, were dissected according to the atlas of Paxinos and Watson (Paxinos and Watson, 2007) from slices of 2-mm-thickness (plates 6–9, including Cg1, Cg3, and IL sub-regions), frozen on dry ice and stored at -80°C. Figure 13 represents a coronal section of the PFC.

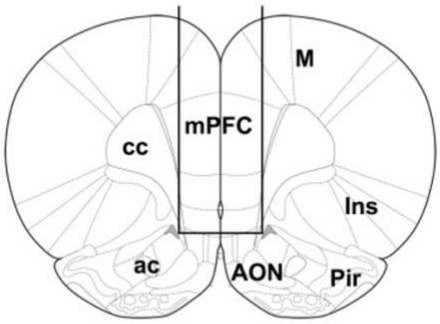

Figure 13: representation of a coronal section of the rat PFC.

3.4. mRNA extraction and gene expression analyses

Total RNA was isolated by a single step of guanidinium isothiocyanate/phenol extraction using PureZol RNA isolation reagent (Bio-Rad Laboratories, Italy) according to the manufacturer's instructions and quantified by spectrophotometric analysis. The samples were then processed for real-time polymerase chain reaction (RT-PCR) to assess the expression of the target genes (primer and probes sequences are listed in Tables 1 and 2). RNA was treated with DNase (Thermoscientific, Italy) to avoid DNA contamination. Gene expression was analyzed by TaqMan qRT-PCR one-step RT-PCR kit for probes (Bio-Rad laboratories, Italy). Samples were run in 384 well formats in triplicate as a multiplexed reaction with a normalizing internal control (*36b4 or β-actin*). 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.

Gene	**Forward sequence**	**Reverse sequence**	**Probe sequence**
Total *Bdnf*	AAGTCTGCATTACATTCCTCGA	GTTTTCTGAAAGAGGGACAGTTTAT	TGTGGTTTGTTGCCGTTGCCAAG
Psd95	CAAGAAATACCGCTACCAAGATG	CCCTCTGTTCCATTCACCTG	TCAACACGGACACCCTAGAAGCC
Cdc42	AAGGCTGTCAAGTATGTGGAG	GCTCTGGAGATGCGTTCATAG	CCTGCGGCTCTTCTTCGGTTCT
Gad65	TGAGGGAAATCATTGGCTGG	TCCCCTTTTCCTTGACTTCTG	TGCCATCTCCAACATGTACGCCA
Gad67	ATACTTGGTGTGGCGTAGC	AGGAAAGCAGGTTCTTGGAG	AAAACTGGGCCTGAAGATCTGTGGT
Vgat	ACGACAAACCCAAGATCACG	GTAGACCCAGCACGAACATG	TTCCAGCCCGCTTCCCACG
Gaba$_A$γ2	ACTCATTGTGGTTCTGTCCTG	GCTGTGACATAGGAGACCTTG	ATGGTGCTGAGAGTGGTCATCGTC
Pvalb	CTGGACAAAGACAAAAGTGGC	GACAAGTCTCTGGCATCTGAG	CCTTCAGAATGGACCCCAGCTCA
Arc	GGTGGGTGGCTCTGAAGAAT	ACTCCACCCAGTTCTTCACC	GATCCAGAACCACATGAATGGG

Gene	Forward sequence	Reverse sequence	Probe sequence
Cfos	TCCTTACGGACTCCCCAC	CTCCGTTTCTCTTCCTCTTCAG	TGCTCTACTTTGCCCCTTCTGCC
Dusp1	TGTGCCTGACAGTGCAGAAT	ATCTTTCCGGGAAGCATGGT	ATCCTGTCCTTCCTGTACCT
Sgk1	GACTACATTAATGGCGGAGAGC	AGGGAGTGCAGATAACCCAAG	TGCTCGCTTCTACGCAGC
Gilz	CGGTCTATCAACTGCACAATTTC	CTTCACTAGATCCATGGCCTG	AACGGAAACCACATCCCCTCCAA
Fkbp5	GAACCCAATGCTGAGCTTATG	ATGTACTTGCCTCCCTTGAAG	TGTCCATCTCCCAGGATTCTTTGGC
P11	AGAGTGCTCATGGAAAGGGA	AGCTCTGGAAGCCCACTTTT	ATAATGAAAGACCTGGACCAGTGC
Foxo1	GAGTGGATGGTGAAGAGTGTG	GGACAGATTGTGGCGAATTG	TCAAGGATAAGGGCGACAGCAACAG
Per1	AGAGCTGAGTCCTTGCCATT	TGGCTGATGACACTGATGCA	AGCGGAGTTCTCACAGTTCA
Per2	TTGTGCCTCCCGATGATGAA	AGTGGGCAGCCTTTCGATTA	GTACATCACACTGGACACTAGC
Cry1	TCAATCCACGGAAAGCCTGT	CCACAAACAACCCACGCTTT	GGAACCCCATCTGTGTTCAA
Cry2	TAGTCCACGCCAATGATGCA	TGCCCAAACTGAAAGGCTTC	TCTATGAGCCCTGGAATGCT
Rev-erbα	ACGTCCCCACACACTTTACA	ACAAGTGGCCATGGAAGACA	GGCACCAGCAACATTACCAA
Rev-erbβ	ACGGATGAGTGTTTCCTGCA	AGCGACGAGGAAATGAGCTT	TTCTGGTGTCTGCAGATCGA
36b4	TCAGTGCCTCACTCCATCAT	AGGAAGGCCTTGACCTTTTC	TGGATACAAAAGGGTCCTGG
β-actin	CACTTTCTACAATGAGCTGCG	CTGGATGGCTACGTACATGG	TCTGGGTCATCTTTTCACGGTTGGC

Table 1: sequences of forward and reverse primers and probes used in RT-PCR analyses and purchased from Eurofins MWG-Operon.

Gene	Accession number	Assay ID
Bdnf long 3'UTR	EF125675.1	Rn02531967_s1
Bdnf isoform IV	EF125679.1	Rn01484927_m1
Bdnf isoform VI	EF125680.1	Rn01484928_m1
Gadd45β	BC085337.1	Rn01452530_g1
Nr4a1	BC097313.1	Rn01533237_m1

Table 2: forward, reverse primers and probes used in RT-PCR analyses and purchased from Life Technologies, which did not disclose the sequences.

3.5. Protein extraction and expression analyses

Western blot analysis was used to investigate the levels of the target proteins in the nuclear fraction, in the crude synaptosomal fraction, in the cytosolic compartment, and the whole homogenate. 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, 1 mM HEPES solution in the presence of a complete set of proteases (Roche) and phosphatase (Sigma-Aldrich) inhibitors. The total homogenate was centrifuged at 1000 g for 10 min at 4°C to obtain a pellet enriched in nuclear components, which was suspended in a buffer [20 mM HEPES, 0.1 mM dithiothreitol (DTT), 0.1 mM EGTA] with protease and phosphatase inhibitors. The supernatant was further centrifuged at 10000 g for 10 min at 4°C to eliminate the membrane fraction and the resulting supernatant corresponding to the cytosolic fraction was conserved for the protein analyses. Total protein content was measured according to the Bradford Protein Assay procedure (Bio-Rad Laboratories), using bovine serum albumin (BSA) as a calibration standard. Equal amounts of protein were run under reducing conditions on 10% SDS-polyacrylamide gels and then electrophoretically transferred onto nitrocellulose membranes (GE Healthcare Life Sciences). The blots were blocked with the proper blocking buffer and then were incubated with the proper primary and secondary antibodies (blocking and antibodies conditions are specified in Table 3). Immunocomplexes were visualized by chemiluminescence using the Chemidoc MP imaging system (Bio-Rad Laboratories). Protein levels were quantified by the evaluation of band densities through ImageLab program (Bio-Rad Laboratories) and normalized to the β-actin.

Protein	Primary antibody	Secondary antibody
mBDNF (Icosagen)	1:1000 BSA 5% Over/Night (O/N) 4°C	Anti-mouse 1:1000 Milk 3% 1h Room Temperature (RT)
MAZ1 (Invitrogen)	1:500 BSA 5% O/N 4°C	Anti-rabbit 1:1000 Milk 3%, 1h RT
SP1 (Invitrogen)	1:250 BSA 5% O/N 4°C	Anti-rabbit 1:1000 Milk 3%, 1h RT
PSD95 (Cell Signalling)	1:4000 BSA 5% O/N 4°C	Anti-rabbit 1:8000 Milk 3% 1h RT
CDC42 (Cell Signalling)	1:1000 BSA 5% O/N 4°C	Anti-rabbit 1:1000 Milk 3% 1h RT
GAD65 (Millipore)	1:2000 Milk 3% O/N 4°C	Anti-rabbit 1:1000 Milk 3% 1h RT
GAD67 (AbCAM)	1:2500 Milk 3% O/N 4°C	Anti-mouse 1:5000 Milk 3% 1h RT
GR (ThermoFisher)	1:500 BSA 5% + 0,2% sodium azide O/N 4°C	Anti-rabbit 1:2000 Milk 3% 1h RT
β-ACTIN (Sigma)	1:10000 Milk 3% 45min RT	Anti-mouse 1:10000 Milk 3% 45 min RT

Table 3: antibodies conditions used in the western blot analyses.

3.6. Plasma corticosterone levels analyses

Blood samples from $TPH2^{+/+}$ and $TPH2^{-/-}$ rats were collected in MiniCollect K3EDTA (Greiner Bio-One GmbH, Frickenhausen, Deutschland) tubes. Plasma was separated by centrifugation for 10 min at 1300 g, at 4°C and corticosterone was determined by an enzyme-linked immunosorbent assay (ELISA) using a commercial kit (IBL International, Hamburg, Germany) according to the manufacturers' instructions.

3.7. HPLC analyses

For blood analyses, three hundred microliters of whole blood were collected into 1 mL syringes prefilled with 100 µL of heparin and quickly transferred to Eppendorf tubes containing 10 µL of perchloric acid (PCA) and 5 µL of 10 mg/mL of ascorbic acid, vortexed, centrifuged (20 000 ×g, 30 minutes, 4°C) and the supernatant was frozen at −80°C until high-sensitive reversed-phase high-performance liquid chromatography (HPLC) analyses.

For the organ collection, rats were transcardially perfused with ice-cold PBS to remove blood, containing platelet 5-HT. Tissues were snap-frozen in liquid nitrogen and kept at −80°C. Frozen tissue samples were homogenized in 710 µM ascorbic acid and 2.4% perchloric acid (Sigma-Aldrich, Steinheim, Germany), and precipitated proteins were pelleted through centrifugation (20 minutes, 20000 g, 4°C) and the collected supernatant was analyzed for serotonergic metabolites (Trp, and 5-HT) using HPLC with fluorometric detection. Samples were separated over a C18 reversed phase column (LipoMare C18, AppliChrom, Oranienburg, and ProntoSIL 120 C18 SH, VDS Optilab, Berlin) at 20°C in a 10 mM potassium phosphate buffer (pH 5.0) (Sigma-Aldrich, Steinheim, Germany) with 5% methanol (Roth, Karlsruhe, Germany) and a flow rate of 0.8-1.0 mL/min. The excitation wavelength was 295 nm and the fluorescent signal was measured at 345 nm. CLASS-VP software (Shimadzu, Tokyo, Japan) was used to analyze the peak parameters of chromatographic spectra and quantify substance levels, based on comparative calculations with alternately measured external standards. Amounts of 5-HT and Trp, were normalized to the wet tissue weight.

3.8. Statistical analyses

All the analyses were conducted by using IBM SPSS Statistics, version 24 or with Microsoft Excel. Results were analyzed with two-way analysis of Variance (ANOVA), three-way ANOVA followed by Fisher's protected least significant difference (PLSD) or with the Student's t-test depending on the experimental groups. Significance for all tests was assumed for $p<0.05$. Data are presented as means±standard error (SEM).

4. Results and discussions

4.1. SERT $^{-/-}$ rats

4.1.1. Introduction

As better explained before, genetic alterations of the serotonin transporter and in particular the presence of a short allele of the promoter region have been associated in humans with an increased risk to develop depression (Caspi et al., 2003; Munafò et al., 2009; Risch et al., 2009; Karg et al., 2011; Sharpley et al., 2014; Bleys et al., 2018; Culverhouse et al., 2018).

The 5-HTTLPR short allele is quite well modeled in rodents by altering the SERT gene transcription thus allowing a deeper study of the lifelong consequences of SERT malfunctioning on behavior and underlying brain circuits. In particular, the SERT$^{-/-}$ rat model was developed in 2004 by Smits and colleagues through the ENU-driven target-selected mutagenesis (Smits et al., 2004, 2006) and it is characterized by a premature stop codon in the third exon (position 3924) that encodes for the second extracellular loop of the transporter. In the whole body, the lack of the transporter in SERT$^{-/-}$ rats results in a massive presence of extracellular serotonin as well as defects in its recycling into the presynaptic membrane. Indeed, the levels of serotonin and of its metabolite, the 5-hydroxyindoleacetic acid, into the presynaptic membrane are dramatically reduced (Homberg et al., 2007).

At behavioral level, SERT$^{-/-}$ rats well mimic a psychopathological condition showing different symptoms typically present in people suffering from these illnesses. Indeed, it has been demonstrated how the absence of the SERT induced an anxiety-like behavior in different behavioral tests both in males as well as in females SERT$^{-/-}$ rats. Accordingly, also the depressive-like behavior is a clear feature of this animal model showing anhedonia and despair (Olivier et al., 2008; Kiser et al., 2012; Mohammad et al., 2016). Consistently, similar results were obtained in SERT$^{-/-}$ mouse models (Holmes et al., 2003a, 2003b; Lira et al., 2003; Alexandre et al., 2006; Zhao et al., 2006; Kalueff et al., 2007).

At the molecular level, it has been demonstrated that SERT deletion induced alterations in different systems that have been linked to mood disorders. Indeed, SERT$^{-/-}$ rats show an impairment in the transcription and translation of the neurotrophin Bdnf, but also in spine markers levels and in the expression of immediate early gene *Activity Regulated Cytoskeleton Associated Protein* (*Arc*) (Molteni et al., 2009b, 2010; Calabrese et al., 2013). Similarly, also the GABAergic and the glutamatergic systems have been found to be altered by the lack of the SERT (Guidotti et al., 2012; Calabrese et al., 2013; Brivio et al., 2019; Schipper et al., 2019).

Hence, SERT$^{-/-}$ rats well mimic the condition of people suffering from psychopathologies both behaviorally and molecularly.

Moreover, SERT$^{+/-}$ rats, which are characterized by milder alterations at basal level from a behavioral and a molecular point of view, showed stronger impairments after negative environmental stimuli such as acute stress during adulthood or prenatal stressors in comparison to their wild-type counterparts (Houwing et al., 2017) supporting the increased susceptibility of people with poor functionality of the transporter in the manifestation of psychiatric symptoms.

Conversely, some evidence showed that SERT knockout rats and mice are more responsive to positive environmental stimuli, for example, to tactile stimulation in early life, conditioned reward in adulthood, housing with a littermate of the opposite sex and enriched environment (Homberg and Lesch, 2011; Nonkes et al., 2012; Kästner et al., 2015; Homberg et al., 2016; Rogers et al., 2017; Roversi et al., 2020). This perfectly fits with the hypothesis that a positive environment can protect against the effects of negative situations and reduce the risk for depression, particularly in early life, but also during adulthood (Kaufman et al., 2004; Belsky et al., 2009; Mitchell et al., 2011; Li et al., 2013; Starr et al., 2013). Indeed, when exposed to supportive environmental stimuli, people carrying the short allele carriers benefit most (Fox et al., 2011; Homberg and Lesch, 2011; Fox and Beevers, 2016). Consistently, a recent theory of differential susceptibility argues that short allele carriers have an increased sensibility to the external conditions with worst outcomes after facing negative conditions, but also best effects after supportive conditions (Belsky et al., 2009; Pluess, 2017).

In light of this evidence and since first-line antidepressant treatments are only partially effective in treating mood disorders (Fava, 2003), possibly because their main target, the SERT, is reduced in those vulnerable to depression (Serretti et al., 2007; Porcelli et al., 2012), non-pharmacological therapies are emerging (Farah et al., 2016; Chen and Shan, 2019). Interestingly, these alternative approaches can also be mimicked at the preclinical level for example by housing the animal in an enriched environment (EE) (Speisman et al., 2013; Gong et al., 2018; Thamizhoviya and Vanisree, 2019).

Because individuals characterized by an inherited reduction in SERT expression seem to be more sensitive to both negative and positive environmental stimuli (Homberg et al., 2016), here, we hypothesized that SERT knockout rats would benefit most from a positive environment in comparison to wild-type animals. In particular, we tested the hypothesis that

the expected pathological-like behavior and the molecular changes of $SERT^{-/-}$ rats could find an amelioration in a condition of positive environment.

Accordingly, it has previously been demonstrated that EE in adulthood reduced anxiety- but not depression-like behavior in SERT knockout mice (Rogers et al., 2017). Here we replicated and extended this study in SERT knockout rats. Specifically, we exposed $SERT^{+/+}$ and $SERT^{-/-}$ male rats to a normal environment (NE) defined as paired housing in standard cages with limited enrichment, or to EE consisting of groups of 10 animals housed in large cages with additional toys, tunnels, and shelters for one month. During this month, the animals were subjected to behavioral tests measuring depression- and anxiety-like behavior. After NE/EE exposure and behavioral testing, $SERT^{-/-}$ animals and their controls were sacrificed to evaluate the effects of environmental manipulation on the expression of Bdnf, GABAergic, and spines markers in the PFC, a brain region that is highly responsive to environmental stimuli and adaptive behavioral responses (Kolb et al., 2012; Brivio et al., 2020b).

4.1.2. Results

4.1.2.1. One Month of Enriched Environment Normalized the Depressive- and Anxiety-Like Behavior in SERT $^{-/-}$ Rats

It has been previously demonstrated that SERT$^{-/-}$ rats display anhedonia and anxiety-like behavior under standard housing conditions (Olivier et al., 2008). Here we tested whether these phenotypes could be normalized by EE. The sucrose consumption test was used to measure anhedonia. As shown in Figure 14A, we found a significant reduction in sucrose preference in SERT$^{-/-}$ rats versus controls under NE conditions (−26% $p<0.05$ vs SERT$^{+/+}$/NE). This phenotype, indicative for anhedonia, was normalized by EE (+22% $p<0.05$ vs SERT$^{-/-}$/NE).

To measure anxiety-like behavior, we subjected the animals to the EPM test. We observed that, compared to wild-type controls, SERT$^{-/-}$ rats, spent less time in the center and in the open arms (−25s $p<0.05$ vs SERT$^{+/+}$/NE) and more time in the closed arms (+25s $p<0.05$ vs SERT$^{+/+}$/NE) of the EPM. Interestingly, these alterations in the time spent in the different places of the maze were not present in SERT$^{-/-}$ animals exposed to the EE (open and center: +16s $p>0.05$ vs SERT$^{-/-}$/NE; closed: −16s $p>0.05$ vs SERT$^{-/-}$/NE) (Figure 14B, C).

Finally, novelty-induced locomotor activity was assessed using the open field test. No changes in activity were observed, neither in genotype groups nor in differential housing groups (Figure 14D, E).

The details of the statistical analyses are listed in Table 4.

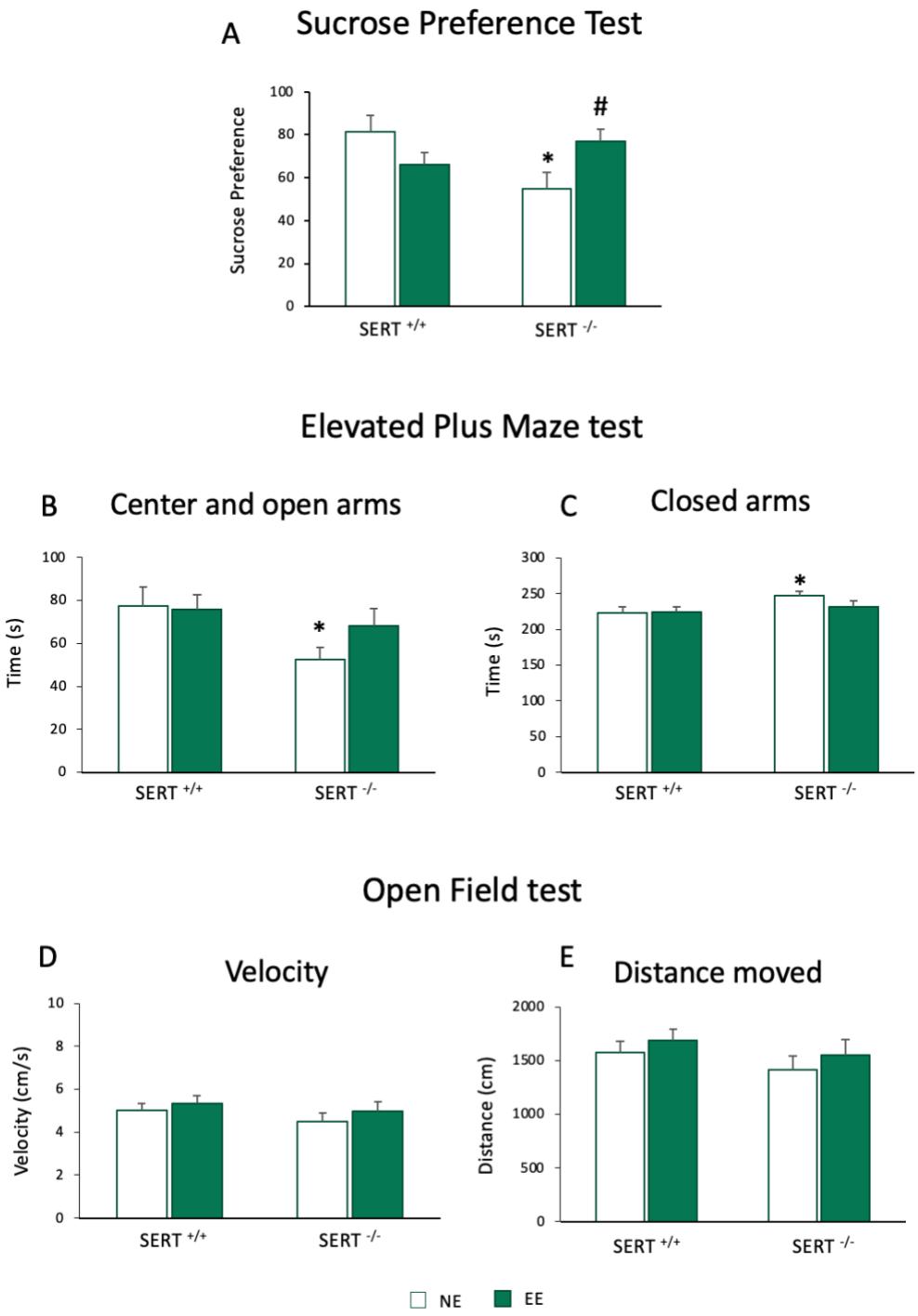

Figure 14: behavioral characterization of SERT$^{+/+}$ and SERT$^{-/-}$ rats subjected to one month of exposure to NE or EE. (A) Sucrose preference in the sucrose consumption test. (B) Time spent in the center and in the open arms (C) and in the closed arms during the EPM test. (D) Velocity in the open field test. (E) moved in the open field test. The data are presented as mean±standard error of the mean (SEM) of at least 7 independent determinations. *p<0.05 vs SERT$^{+/+}$/NE; #p<0.05 vs SERT$^{-/-}$/NE; two-way ANOVA followed by Fisher's PLSD.

4.1.2.2. The EE Improved Neuroplastic Mechanisms in SERT$^{-/-}$ Rats

Since it has been previously demonstrated that SERT$^{-/-}$ rats display impairments in the expression of the neuroplasticity marker Bdnf in the prefrontal cortex, both at mRNA and protein level (Calabrese et al., 2013), we evaluated if EE would alter Bdnf transcription and translation.

As shown in Figure 15A, B, the significant reduction in mBDNF protein levels found in SERT$^{-/-}$ rats (−48% $p<0.05$ vs SERT$^{+/+}$/NE) was partially restored by the EE (+41% $p>0.05$ vs SERT$^{-/-}$/NE). In line, we found a similar effect at the transcriptional level. Indeed, we observed a slight reduction in the transcription of the *Bdnf* long pool of transcripts in SERT$^{-/-}$ rats versus controls under normal housing conditions (−20% $p>0.05$ vs SERT$^{+/+}$/NE) and an-upregulation in EE exposed SERT$^{-/-}$ rats (+50% $p<0.05$ vs SERT$^{-/-}$/NE) (Figure 15D). Moreover, we found an upregulation of total *Bdnf* expression in SERT$^{-/-}$ rats exposed to the positive housing conditions (+50% $p<0.05$ vs SERT$^{-/-}$/NE) (Figure 15C).

The details of the statistical analyses are listed in Table 4.

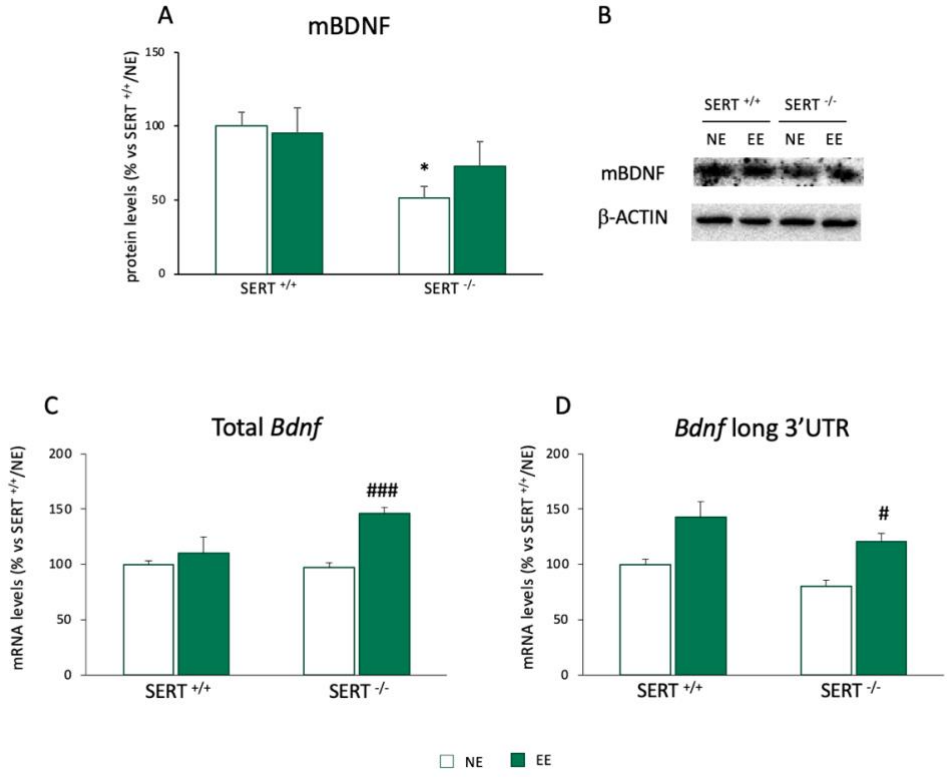

Figure 15: analyses of mBDNF protein levels (A-B) and of total *Bdnf* (C) and *Bdnf* long 3'UTR (D) mRNA levels in the PFC of SERT$^{+/+}$ and SERT$^{-/-}$ rats subjected to one month of exposure to NE or EE. The data are presented as percent change of SERT$^{+/+}$/NE and are expressed as mean±standard error of the mean (SEM) of at least 5 independent determinations for western blot analyses and of 9 for RT-PCR analyses. *$p<0.05$ vs SERT$^{+/+}$/NE; #$p<0.05$, ###$p<0.001$ vs SERT$^{-/-}$/NE; two-way ANOVA with Fisher's PLSD.

4.1.2.3. The Reduction of Spine Markers Expression in SERT$^{-/-}$ Rats Is Normalized by EE

As a proxy of spine functionality, we measured the expression of some markers of dendritic spine densities to assess a possible positive effect of EE in SERT$^{-/-}$ rats versus wild-type counterparts.

As shown in Figure 16, we found a downregulation in postsynaptic density-95 (PSD95) (Figure 16A, C) and Cell Division Cycle 42 (CDC42) (Figure16B, C) protein levels (PSD95: −32% p<0.01 vs SERT$^{+/+}$/NE; CDC42: −42% p<0.01 vs SERT$^{+/+}$/NE) in SERT$^{-/-}$ rats compared to wild-type rats, while EE normalized their levels (PSD95: +40% p<0.01 vs SERT$^{-/-}$NE; CDC42: +80% p<0.01 vs SERT$^{-/-}$/NE).

In line with the protein expression data, mRNA expression levels of *Psd95* were significantly reduced in SERT$^{-/-}$ rats (−15% p<0.05 vs SERT$^{+/+}$/NE) but normalized by EE exposure (+23% p<0.01 vs SERT$^{-/-}$/NE) (Figure 16D). Similarly, we found a slight reduction in *Cdc42* mRNA levels in SERT$^{-/-}$ rats (−9% p>0.05 vs SERT$^{+/+}$/NE) and a trend to an increase in SERT$^{-/-}$ rats exposed to EE (+20% p>0.05 vs SERT$^{-/-}$/NE) (Figure 16E).

The details of the statistical analyses are listed in Table 4.

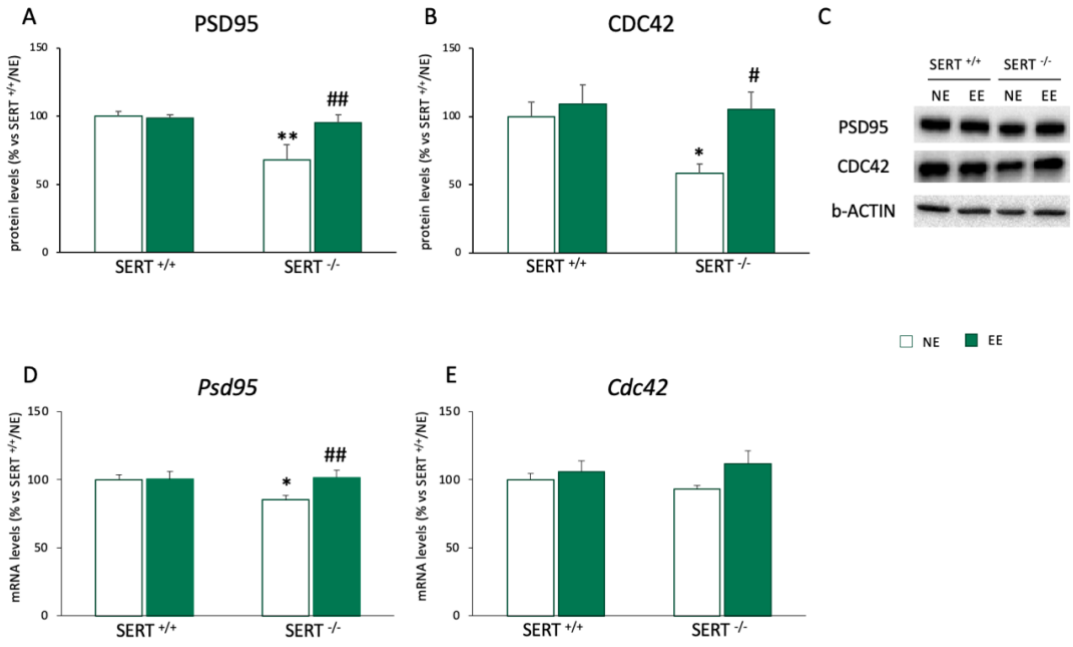

Figure 16: analyses of PSD95 and CDC44 protein levels (A-B-C) and mRNA levels (D-E) in the PFC of SERT$^{+/+}$ and SERT$^{-/-}$ rats subjected to one month of exposure to NE or EE. The data are presented as percent change of SERT$^{+/+}$/NE and are expressed as mean±standard error of the mean (SEM) of at least 4 independent determinations for western blot analyses and of 9 for RT-PCR analyses. *p<0.05, **p<0.01 vs SERT$^{+/+}$/NE; #p<0.05, ##p<0.01 vs SERT$^{-/-}$/NE; two-way ANOVA with Fisher's PLSD.

4.1.2.4. GABAergic System Alterations of SERT$^{-/-}$ Rats Are Restored by the EE

The major inhibitory neurotransmitter in the brain is GABA, and alterations in its system are often linked to anxiety (Kalueff and Nutt, 2007). Given the normalization of anxiety-like behavior in EE versus NE exposed SERT$^{-/-}$ animals, we analyzed the expression levels of GAD65 and GAD67, which are responsible for the production of GABA in the brain. As shown in Figure 17A, B, C, we found a downregulation of both Glutamate Decarboxylase 65 (GAD65) and Glutamate Decarboxylase 67 (GAD67) in SERT$^{-/-}$ rats (GAD65: −43% $p<0.05$ vs SERT$^{+/+}$/NE; GAD67: −39% $p<0.05$ vs SERT$^{+/+}$/NE) which was normalized by EE (GAD65: +87% $p<0.05$ vs SERT$^{-/-}$/NE; +53% $p>0.05$ vs SERT$^{-/-}$/NE).

In line, the mRNA levels of *Gad65* and *Gad67* were downregulated in SERT$^{-/-}$ rats (*Gad65*: −21% $p<0.05$ vs SERT$^{+/+}$/NE; *Gad67*: −19% $p<0.05$ vs SERT$^{+/+}$/NE) and these changes were normalized by the EE (*Gad65*: +39% $p<0.01$ vs SERT$^{-/-}$/NE; *Gad67*: +15% $p>0.05$ vs SERT$^{-/-}$/NE). Moreover, we found an increase in *Gad67* expression in SERT$^{+/+}$ animals with EE (+21% $p<0.05$ vs SERT$^{+/+}$/NE) (Figure 17D, E).

Finally, as shown in Figure 17F, G, H, we found in SERT$^{-/-}$ rats compared to wild-type controls a downregulation in the expression of the vesicular GABA transporter (*Vgat)*, the GABA type A receptor subunit alpha2 (*GABA$_A\gamma 2$)*, and of the GABAergic interneurons marker Parvalbumin (*Pvalb)* (*Vgat*: −33% $p<0.05$ vs SERT$^{+/+}$/NE; *GABA$_A\gamma 2$*: −17% $p<0.05$ vs SERT$^{+/+}$/NE; *Pvalb*: −20% $p<0.05$ vs SERT$^{+/+}$/NE). These down-regulations were normalized by EE (*Vgat*: +13% $p>0.05$ vs SERT$^{-/-}$/NE; *GABA$_A\gamma 2$*: +20% $p<0.05$ vs SERT$^{-/-}$/NE; *Pvalb*: +34% $p<0.01$ vs SERT$^{-/-}$/NE).

The details of the statistical analyses are listed in Table 4.

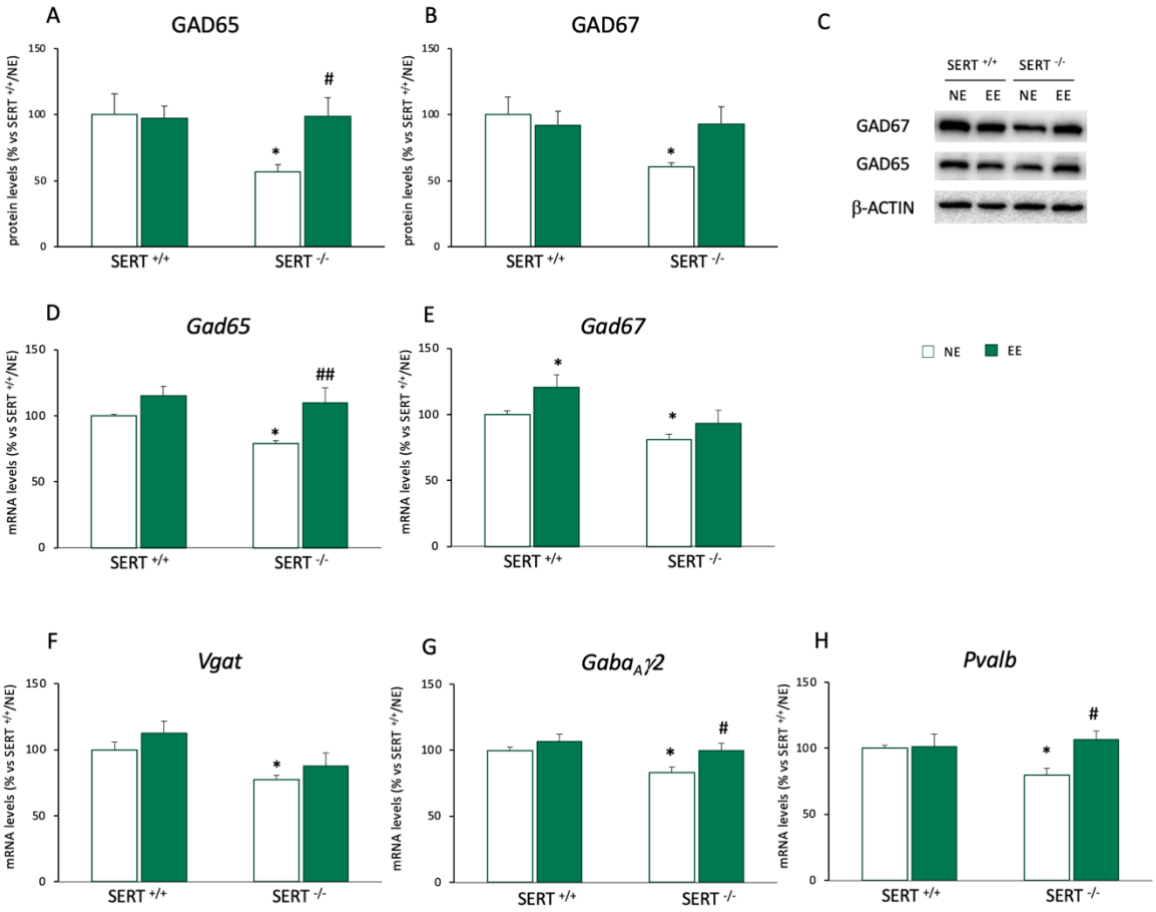

Figure 17: analyses of GAD65 and GAD67 protein levels (A-B-C) and of *Gad65*, *Gad67*, *Pvalb*, *Gaba$_A\gamma$2* and *Vgat* mRNA levels (D-E-F-G-H) in the PFC of SERT$^{+/+}$ and SERT$^{-/-}$ rats subjected to one month of exposure to NE (normal environment) or EE (environmental enrichment). The data are presented as percent change of SERT$^{+/+}$/NE and are expressed as mean±standard error of the mean (SEM) of at least 4 independent determinations for western blot analyses and of 8 for RT-PCR analyses. *p<0.05 vs SERT$^{+/+}$/NE; #p<0.05, ##p<0.01 vs SERT$^{-/-}$/NE; two-way ANOVA with Fisher's PLSD.

Protein/Gene	two-way ANOVA
sucrose preference	genotype X EE interaction: $F_{(1,30)}$ =7.463 p<0.05
time in the center and in the open arms	genotype: $F_{(1,35)}$ = 4.904 p<0.05
time in the closed arms	genotype: $F_{(1,35)}$ = 4.904 p<0.05
mBDNF	genotype: $F_{(1,21)}$=46.266 p<0.05
Total *Bdnf*	genotype X EE interaction: $F_{(1,37)}$=4.930 p<0.05
Bdnf long 3'UTR	EE: $F_{(1,38)}$=7.927 p<0.01
PSD95	genotype: $F_{(1,38)}$=8.923 p<0.01 EE: $F_{(1,38)}$=4.604 p<0.05 genotype X EE: $F_{(1,38)}$=5.591 p<0.05
CDC42	EE: $F_{(1,38)}$=4.718 p<0.05
Psd95	EE: $F_{(1,38)}$=4.931 p<0.05; genotype X EE interaction: $F_{(1,38)}$=4.641 p<0.05
Cdc42	Not Significant
GAD65	genotype X EE: $F_{(1,18)}$=5.852 p<0.05
GAD67	genotype X EE: $F_{(1,18)}$=2.919 p>0.05
Gad65	EE: $F_{(1,38)}$=10.242 p<0.01
Gad67	genotype: $F_{(1,35)}$=11.422 p<0.01 EE: $F_{(1,35)}$=5.767 p<0.05
Pvalb	EE: $F_{(1,37)}$=4.537 p<0.05 genotype X EE: $F_{(1,37)}$=3.909 p=0.056
Vgat	genotype: $F_{(1,37)}$=10.172 p<0.01
$GABA_A$ g2	genotype: $F_{(1,38)}$=6.149 p<0.05 EE: $F_{(1,38)}$=5.842 p<0.05;

Table 4: statistical analyses details of the behavioral and molecular analyses of $SERT^{+/+}$ and $SERT^{-/-}$ rats subjected to one month of exposure to NE or EE.

4.1.3. Discussion

SERT$^{-/-}$ rats are one of the most employed animal models to study mechanisms related to vulnerability to depression, since they display different features of the human illness both at a behavioral and molecular level (Olivier et al., 2008; Calabrese et al., 2010a; Molteni et al., 2010; Guidotti et al., 2012; Brivio et al., 2019; Schipper et al., 2019).

By using this animal model, here we confirmed the depressive- and anxiety-like phenotypes of SERT$^{-/-}$ rats, as well as the impairments, at molecular level, of the neurotrophic factor Bdnf, and the spine and GABAergic markers (Guidotti et al., 2012; Calabrese et al., 2013; Brivio et al., 2019). Interestingly, we observed that one month of EE exposure normalized these alterations both at a behavioral and molecular level. These findings confirm our hypothesis that deleting the SERT induces an increased sensibility to the external environment supporting the idea that a stimulating situation can have a beneficial impact on SERT$^{-/-}$ rats.

In the sucrose consumption test, we found a reduced preference of the sucrose solution relative to plain water, confirming the anhedonic phenotype of this animal model (Olivier et al., 2008). Similarly, in line with the literature results (Olivier et al., 2008; Golebiowska et al., 2019), we found anxiety-like behavior in SERT$^{-/-}$ rats tested in the EPM test. Interestingly, one month of EE normalized these anxiety and depressive-like symptoms in SERT$^{-/-}$ rats. Accordingly, it has been shown that exposure to positive stimuli, such as the EE or physical exercise, normalized the pathological phenotype in different animal models of depression (Patki et al., 2014; Gong et al., 2018; Su et al., 2020) suggesting that a constructive environment might help in ameliorating human traits of mood disorders. Notably, we found a specific effect of the EE on SERT$^{-/-}$ rats, while no behavioral improvements in SERT$^{+/+}$, in line with the major sensitivity for external stimuli when SERT functionality is reduced (Homberg et al., 2016).

Seen the restorative effect of the EE at a behavioral level, we decided to deepen the molecular mechanisms, possibly underlying the positive outcome of EE. In particular, since we previously demonstrated that SERT$^{-/-}$ rats are characterized by an impairment in neuroplastic mechanisms (Calabrese et al., 2013), we measured *Bdnf* transcription and translation. In line with previous results (Calabrese et al., 2013), we found reduced mBDNF protein levels, as well as of the pool of the long transcripts of Bdnf in SERT$^{-/-}$ rats with a normalization, due to EE exposure. This perfectly fits the increase in neurotrophin levels after positive environmental

stimuli that are often paralleled by improvements at a behavioral level (Dong et al., 2018; Brivio et al., 2020b).

One of the roles of Bdnf includes the promotion of spine maturation and formation (An et al., 2008; Kaneko et al., 2012; Orefice et al., 2013), for instance, in animal models of depression (McEwen, 2005; Ren et al., 2015; Brivio et al., 2019). Here, along with our previous data (Brivio et al., 2019), we found a reduction in the spine markers PSD95 and CDC42 in NE exposed SERT$^{-/-}$ rats. Like for Bdnf, one month of EE was able to normalize their expression. Interestingly, it has been demonstrated that antidepressant treatments normalized spine atrophy and density reduction in animal models of depression (Norrholm and Ouimet, 2001; Duman and Duman, 2014), suggesting that also EE, by increasing these spine markers expression, could have a restorative effect on spine morphology.

Finally, given the tight co-play between the GABA system and anxious behavior (Kalueff and Nutt, 2007), we analyzed the expression of GABAergic markers previously found to be impaired in SERT$^{-/-}$ rats (Guidotti et al., 2012). Interestingly, we found a reduction in the expression of GAD65 and GAD67, enzymes responsible for the production of the inhibitory neurotransmitter, as well as of the vesicular transporter *Vgat*. Moreover, in SERT$^{-/-}$ animals, we found a reduction in *Pvalb*, a GABAergic interneurons marker (Toledo-Rodriguez et al., 2005), as well as in *GABA$_A\gamma$2*, the most abundant GABA receptor subunit in the adult brain which deletion promotes an anxiety-like behavior in rodents (De Blas, 1996; Crestani et al., 1999). Furthermore, in line with the results obtained from the EPM test, all these GABAergic alterations were normalized by EE, further supporting the positive impact of enriched housing in SERT$^{-/-}$ rats.

Taken the behavioral and molecular data together, we observed a specific effect of EE on SERT$^{-/-}$ rats, while SERT$^{+/+}$ animals seemed to be unaffected by the environmental manipulation. This is in line with the vantage sensitivity theory stating that some plasticity factors, like the 5-HTTLPR s-allele disproportionally benefit sensitive individuals (Pluess, 2017). Individuals not carrying such plasticity factors, on the other hand, remain largely unaffected by positive environments. The differential susceptibility theory states that individuals carrying plasticity factors are sensitive to both positive and negative stimuli (Belsky et al., 2009). As mentioned before, there is extensive evidence that the SERT gene behaves according to this latter theory. However, a limitation of the present study is that we did not expose the animals to a negative environment, like social isolation, to investigate whether behavioral and molecular

parameters would worsen compared to the normal housing condition. Moreover, seen the different impact of the EE in males and females and the prevalence of the pathology in women (Van de Velde et al., 2010; Chourbaji et al., 2012), we think that further studies employing female rats could improve our results and highlight possible differences in between the two sexes at basal level and in response to the positive stimuli.

In summary, our data confirm that the behavioral and molecular characterization of $SERT^{-/-}$ rats at basal level. Moreover, it also provides interesting new insights on the possible use of non-pharmacological approaches to be employed as supportive therapies to treat psychopathologies, improving patients' compliance, and increasing the successful treatment rate.

4.2. TPH2 $^{-/-}$ rats

4.2.1. Introduction

Alterations in serotonin concentration have been reported to be present in patients with different mood disorders (Brewerton, 1995). In line, genetic mutations of the gene coding for the rate-limiting enzyme for the production of serotonin in the central nervous system have been related in humans to episodes of psychiatric illnesses (Zhang et al., 2005; Russo et al., 2007; Cichon et al., 2008; McKinney et al., 2009; Popova and Kulikov, 2010; Waider et al., 2011; Ma et al., 2015) as well as of cognitive deficits (Strobel et al., 2007). However, other studies did not find a correlation between TPH2 alterations and psychopathologies (Khabour et al., 2013; Laksono et al., 2019).

Hence, to better understand and characterize the effect of the malfunctioning of the TPH2 gene in the field of psychiatric disorders, TPH2 knock-out rodents have been developed. In particular, TPH2$^{-/-}$ rats were generated through the zinc finger nucleases technique on a Dark Agouti background by Kaplan and colleagues in 2016 resulting in no detectable TPH2 immunoreactivity in the brain while peripheral levels of the serotonin that were not altered (Kaplan et al., 2016). Despite the absence of serotonin in the central nervous system, serotonergic neuron formation and maturation as well as the levels of serotonergic markers other than TPH2 were not affected by the deficiency of the neurotransmitter (Gutknecht et al., 2008, 2012). Finally, norepinephrine and dopamine levels were comparable between genotypes (Kaplan et al., 2016) while GABA and glutamate were increased in the hippocampus and in the PFC of mice lacking central serotonin (Waider et al., 2013).

Interestingly, TPH2$^{-/-}$ rats showed an increased mortality, a growth retardation during the pre-adolescent period despite the normal feeding behavior and normal maternal-care behavior (Kaplan et al., 2016). Moreover, at behavioral level rodents lacking central serotonin demonstrated an increased aggressivity but also a decreased anxiety (Mosienko et al., 2012; Peeters et al., 2019). Hence, this animal model does not show a clear pathological phenotype at behavioral level. However, less is known on the molecular alterations induced by the lack of serotonin at central level. Therefore, considering the strict crosstalk between the serotonergic system and the neuroplastic mechanisms (Homberg et al., 2014), here we measured the expression of the most abundant neurotrophin in the brain, Bdnf, in TPH2 deficient rats evaluating also possible differences between the two sexes. Moreover,

serotonin is implicated in developmental processes and during the first stages of life it acts also as a trophic factor for brain maturation playing a central role in cell proliferation and differentiation (Azmitia, 2001; Buznikov et al., 2001; Gaspar et al., 2003). Hence, we decided to analyze if there were alterations in *Bdnf* expression due to the lack of serotonin production in the brain during the first stages of life focusing on three time points from birth until early adolescence.

Finally, to evaluate whether hyposerotoninergia in the brain could affect the response to an acute challenge, we exposed $TPH2^{-/-}$ and $TPH2^{+/+}$ adult rats to one single session of acute restraint stress. Indeed, while long periods of stress have a negative impact on the organism both on behavior and on molecular pathways acting as precipitating factors for psychopathologies, short periods of stress induce, in healthy subjects, the activation of different systems required to cope with the stress (McEwen et al., 2015). However, the presence of a risk factor such as genetic alterations in the serotonergic system may prevent these active mechanisms leading to maladaptive responses that could ultimately lead to a pathological condition. Considering the strong relationship between acute stress and neuroplasticity as well as the involvement of the serotonin in the regulation of Bdnf during acute challenges (Calabrese et al., 2009; Foltran and Diaz, 2016), we evaluated *Bdnf* modulation in these animals lacking central serotonin. Furthermore, the hypothalamic-pituitary-adrenal (HPA) axis is another system physiologically activated to cope with acute stressors (McEwen, 2007) and here we evaluated if the absence of central serotonin could prevent its activation focusing on the genomic pathway that occurs when the glucocorticoid receptor (GR) is internalized into the nucleus and acts as transcription factor for some genes named glucocorticoid responsive genes by binding to specific DNA sequences named glucocorticoid responsive elements (GRE) (Trapp et al., 1994). Interestingly, some of the genes involved in the regulation of the circadian rhythms are also responsive to the glucocorticoids and their expression is modulated by acute stress and corticosterone release as well (Yamamoto et al., 2005; Nader et al., 2010). Therefore, we also analyzed the gene expression profile of genes responsible for the control of clock gene machinery.

As for the $SERT^{-/-}$ rats, all the analyses were conducted in the PFC, a brain region connected to serotonin function and affected by stress exposure (Duman and Monteggia, 2006; Stuss and Knight, 2009; Brivio et al., 2020b).

4.2.2. Results

4.2.2.1. Bdnf expression was upregulated in the PFC TPH2$^{-/-}$ male and female adult rats

We first investigated whether the mRNA expression of total *Bdnf* was modulated by the lack of serotonin, in the PFC of both male and female adult rats. As shown in Figure 18A, we found a significant effect of the genotype ($F_{(1–52)}$ = 10.232, p<0.01; two-way ANOVA) on total *Bdnf* mRNA expression with an upregulation both in TPH2$^{-/-}$ male rats (+23%, p<0.05 vs TPH2$^{+/+}$; Fisher's PLSD) and in TPH2$^{-/-}$ female rats (+25%, p<0.05 vs TPH2$^{+/+}$; Fisher's PLSD). To assess if the changes in total *Bdnf* mRNA were paralleled by alterations in BDNF protein, we investigated the levels of the mature form of BDNF (mBDNF) in crude synaptosomal fraction. Interestingly, we found a significant effect of the genotype ($F_{(1–27)}$ = 17.673, p<0.001; two-way ANOVA) on the levels of mBDNF that were significantly increased in both the sexes (male: +78%, p<0.05 vs. TPH2$^{+/+}$/male; Fisher's PLSD; female: +139%, p<0.01 vs TPH2$^{+/+}$/female; Fisher's PLSD) TPH2$^{-/-}$ rats in comparison to the TPH2$^{+/+}$counterparts (Figure 18B).

Based on the enhancement of total *Bdnf* levels found in TPH2$^{-/-}$ rats, we decided to evaluate if serotonin-deficiency in the CNS differently affects the expression of the major *Bdnf* transcripts. Specifically, we quantified the expression levels of long 3'UTR *Bdnf* transcripts, and isoforms IV and VI to establish their contribution to the observed modulation in total *Bdnf*. *Bdnf* long 3'UTR was significantly modulated by the genotype ($F_{(1–53)}$ = 4.703, p<0.05; two-way ANOVA), with an up-regulation of *Bdnf* long 3'UTR mRNA levels specifically in male TPH2$^{-/-}$ in comparison to TPH2$^{+/+}$ rats (+28%, p<0.05 vs TPH2$^{+/+}$/male; Fisher's PLSD). Moreover, we found a significant effect of the sex ($F_{(1–53)}$ = 5.884, p<0.05; two-way ANOVA) with an increased expression of *Bdnf* long 3'UTR in female TPH2$^{+/+}$ in comparison to male counterparts (+30%, p<0.05 vs TPH2$^{+/+}$/male; Fisher's PLSD; Figure 18C). Similar to the total form of the neurotrophin, we observed a significant effect of the genotype ($F_{(1–54)}$ = 14.017, p<0.05; two-way ANOVA) for *Bdnf* isoform IV (Figure 18D). Accordingly, we found a significant upregulation of *Bdnf* IV expression in TPH2$^{-/-}$ of both sexes (male: +39%, p<0.01 vs TPH2$^{+/+}$/male; female: +27%, p<0.05 vs. TPH2$^{+/+}$/female; Fisher's PLSD). On the contrary, as shown in Figure 18E, we found a significant genotype X sex interaction ($F_{(1–53)}$ = 4.580, p<0.05; two-way ANOVA) for *Bdnf* isoform VI. Indeed, *Bdnf* VI was increased only in male TPH2$^{-/-}$ rats (+22%, p<0.05 vs TPH2$^{+/+}$/male; Fisher's PLSD).

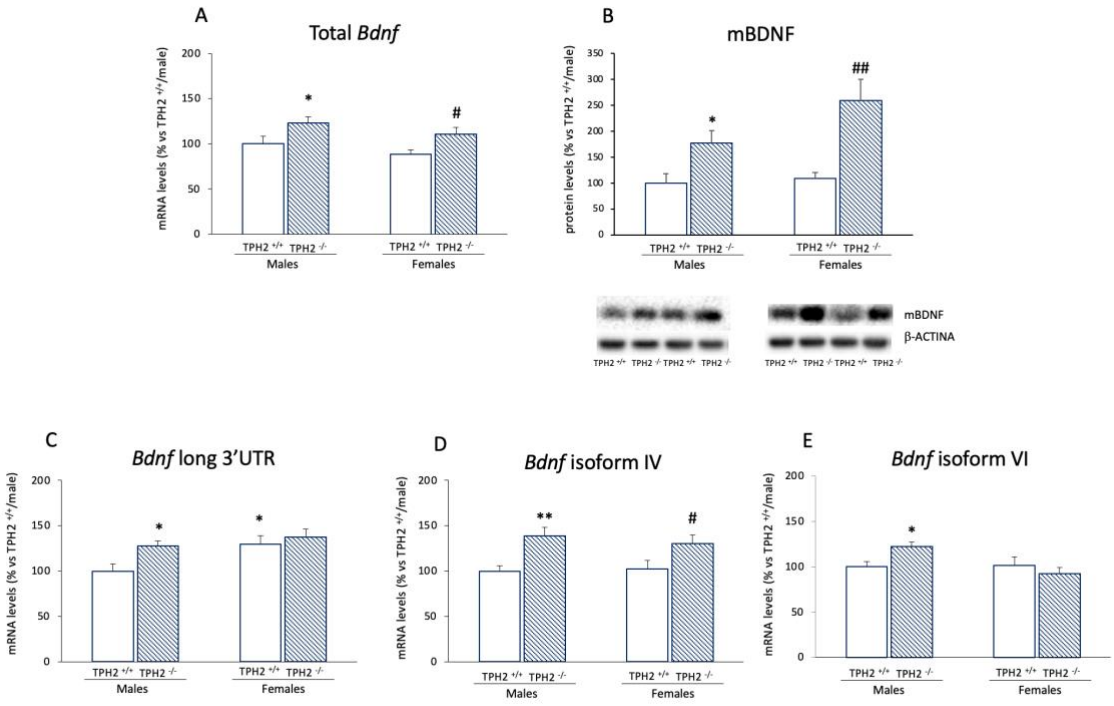

Figure 18: analyses of total *Bdnf* mRNA levels (A), mBDNF protein levels (B) and of *Bdnf* long 3'UTR (C), *Bdnf* isoform IV (D) and VI (E) mRNA levels in the PFC of $TPH2^{+/+}$ and $TPH2^{-/-}$ male and female adult rats. The data are presented as percent change of $TPH2^{+/+}$/male and are expressed as mean±standard error of the mean (SEM) of at least 6 independent determinations for western blot analyses and of 14 for RT-PCR analyses. *p<0.05; **p<0.01 vs $TPH2^{+/+}$/male; #p<0.05, ##p<0.01 vs $TPH2^{+/+}$/female; two-way ANOVA with Fisher's PLSD.

4.2.2.2. The upregulation of Bdnf levels in the frontal lobe of TPH2$^{-/-}$ male and female started from early adolescent while it was not present during early post-natal period

To evaluate the impact of serotonin deficiency during development on *Bdnf* expression, we investigated its mRNA levels in TPH2$^{-/-}$ and TPH2$^{+/+}$ rats at different ages of life from birth until early adolescence, specifically at PND 1, 10 and 30.

In male rats, we found a significant effect of the age ($F_{(2–43)}$ = 107.444, p<0.001; two-way ANOVA) that is reflected by a significant increase of total *Bdnf* transcripts in both genotypes, at PND10 compared to PND1 rats (TPH2$^{+/+}$/PND10: +1657%, p<0.001 vs TPH2$^{+/+}$/PND1; TPH2$^{-/-}$ /PND10: +1467%, p<0.01 vs. TPH2$^{-/-}$/PND1; Fisher's PLSD) but also at PND30 compared to PND10 animals (TPH2$^{+/+}$ /PND30: +59%, p<0.001 vs TPH2$^{+/+}$/PND10; TPH2$^{-/-}$/PND30: +240%, p<0.001 vs TPH2$^{-/-}$/PND10; Fisher's PLSD; Figure 19A). Moreover, we found a significant age X genotype interaction ($F_{(2–43)}$ = 7.115, p<0.01; two-way ANOVA). Indeed, the developmental profiles of the *Bdnf* expression differed between the genotypes, with an equal expression at PND1, decreased total *Bdnf* mRNA levels in TPH2$^{-/-}$ at PND10 (-43%, p<0.01 vs TPH2$^{+/+}$/PND10; Fisher's PLSD), and a significant increase at PND30 (+22%, p<0.05 vs TPH2$^{+/+}$/PND30; Fisher's PLSD). Similarly, we observed a significant effect of the age ($F_{(2–45)}$ = 297.514, p<0.001; two-way ANOVA) for *Bdnf* long 3'UTR transcripts with an upregulation from PND10 to PND30 in both TPH2$^{+/+}$ (TPH2$^{+/+}$/PND30: +1315%, p<0.001 vs TPH2$^{+/+}$/PND10; Fisher's PLSD) and TPH2$^{-/-}$ rats (TPH2$^{-/-}$/PND30: +1355%, p<0.001 vs TPH2$^{-/-}$ /PND10; Fisher's PLSD; Figure 19B). Moreover, we found a significant effect of age ($F_{(2–46)}$ = 287.421, p<0.001; two-way ANOVA) for *Bdnf* isoform IV, with an increase in both genotypes from PND10 to PND30 (TPH2$^{+/+}$/pnd30: +940%, p<0.001 vs TPH2$^{+/+}$/PND10; TPH2$^{-/-}$/PND30: +1587%, p<0.001 vs TPH2$^{-/-}$/PND10; Fisher's PLSD). Furthermore, there was a significant effect of the genotype ($F_{(1–46)}$ = 8.667, p<0.01; two-way ANOVA) and a significant genotype X age interaction ($F_{(2–46)}$ = 10.602, p<0.001; two-way ANOVA) with an upregulation of *Bdnf* isoform IV at PND30 in TPH2$^{-/-}$ rats (+43%, p<0.001 vs TPH2$^{+/+}$/PND30; Fisher's PLSD; Figure 19C). Similarly, *Bdnf* isoform VI was significantly modulated by the age ($F_{(2–46)}$ = 823.232, p<0.001; two-way ANOVA) with an increase both in TPH2$^{+/+}$ and TPH2$^{-/-}$ from PND1 to PND10 (TPH2$^{+/+}$ /PND10: +486, p<0.01 vs TPH2$^{+/+}$ /PND1; TPH2$^{-/-}$/PND10: +473, p<0.05 vs TPH2$^{-/-}$/PND1; Fisher's PLSD) and also from PND10 to PND30 (TPH2$^{+/+}$/PND30: +570%, p<0.001 vs. TPH2$^{+/+}$ /PND10; TPH2$^{-/-}$/PND30:

+1072%, p<0.001 vs TPH2$^{-/-}$/PND10; Fisher's PLSD). Moreover, we found a significant effect of the genotype ($F_{(1–46)}$ = 10.912, p<0.01; two-way ANOVA) and a significant genotype X age interaction ($F_{(2–46)}$ = 19.039, p<0.001; two-way ANOVA) with an upregulation of *Bdnf* isoform VI only at PND30 in TPH2$^{-/-}$in comparison to TPH2$^{+/+}$ rats of the same age (+30%, p<0.001 vs TPH2$^{+/+}$/PND30; Fisher's PLSD; Figure 19D).

As shown in Figure 19E, in female rats, we found a significant effect of the age ($F_{(2–41)}$ = 105.889, p<0.001; two-way ANOVA) for total *Bdnf* mRNA levels with a significant increase among the three ages. In line, we observed an enhancement from PND1 to PND10 only in TPH2$^{-/-}$rats (TPH2$^{-/-}$/PND10: +1624%, p<0.01 vs TPH2$^{-/-}$/PND1; Fisher's PLSD), while starting from PND10, an upregulation of total *Bdnf* mRNA levels was seen in both genotypes (TPH2$^{+/+}$/PND30: +170%, p<0.001 vs TPH2$^{+/+}$/PND10; TPH2$^{-/-}$/PND30: +241%, p<0.001 vs TPH2$^{-/-}$/PND10; Fisher's PLSD). Moreover, we found a significant increase of total *Bdnf* in TPH2$^{-/-}$ rats compared to TPH2$^{+/+}$ (+64%, p<0.001 vs TPH2$^{+/+}$/ PND30; Fisher's PLSD) only at PND30, as supported by the significant effect of the genotype ($F_{(1–41)}$ = 8.400, p<0.01; two-way ANOVA) and of the interaction age X genotype ($F_{(2–41)}$ = 9.843, p<0.001; two-way ANOVA). With respect to *Bdnf* long 3'UTR (Figure 19F), we observed a significant effect of the age ($F_{(2–43)}$ = 292.426, p<0.001; two-way ANOVA; two-way ANOVA) with an upregulation at PND30 compared to PND10 in both TPH2$^{+/+}$ (TPH2$^{+/+}$/PND30: +1601%, p < 0.001 vs TPH2$^{+/+}$/PND10; Fisher's PLSD) and TPH2$^{-/-}$ (TPH2$^{-/-}$/PND30: +1210%, p<0.001 vs TPH2$^{-/-}$/PND10; Fisher's PLSD) . Similarly, *Bdnf* isoform IV was significantly modulated by the age ($F_{(2–43)}$ = 217.038, p<0.001; two-way ANOVA), in young female rats. Accordingly, we observed an increase in both genotypes from PND10 to PND30 (TPH2$^{+/+}$ /PND30: +1484%, p<0.001 vs TPH2$^{+/+}$/PND10; TPH2$^{-/-}$/PND30: +1676%, p<0.001 vs TPH2$^{-/-}$/PND10; Fisher's PLSD). Furthermore, the lack of serotonin at PND30 induced an upregulation of *Bdnf* isoform IV in comparison to TPH2$^{+/+}$/PND30 (+25%, p<0.01 vs TPH2$^{+/+}$/PND30; Figure 19G). In line with what was observed for *Bdnf* long 3'UTR transcripts, *Bdnf* isoform VI was significantly modulated by the age ($F_{(2–42)}$ = 535.298, p<0.001; two-way ANOVA) with the increase, both in TPH2$^{+/+}$ and TPH2$^{-/-}$, from PND10 to PND30 (TPH2$^{+/+}$ /pnd30: +2105%, p<0.001 vs TPH2$^{+/+}$ /PND10; TPH2$^{-/-}$/PND30: +5705%, p<0.001 vs TPH2$^{-/-}$/PND10; Fisher's PLSD; Figure 19H).

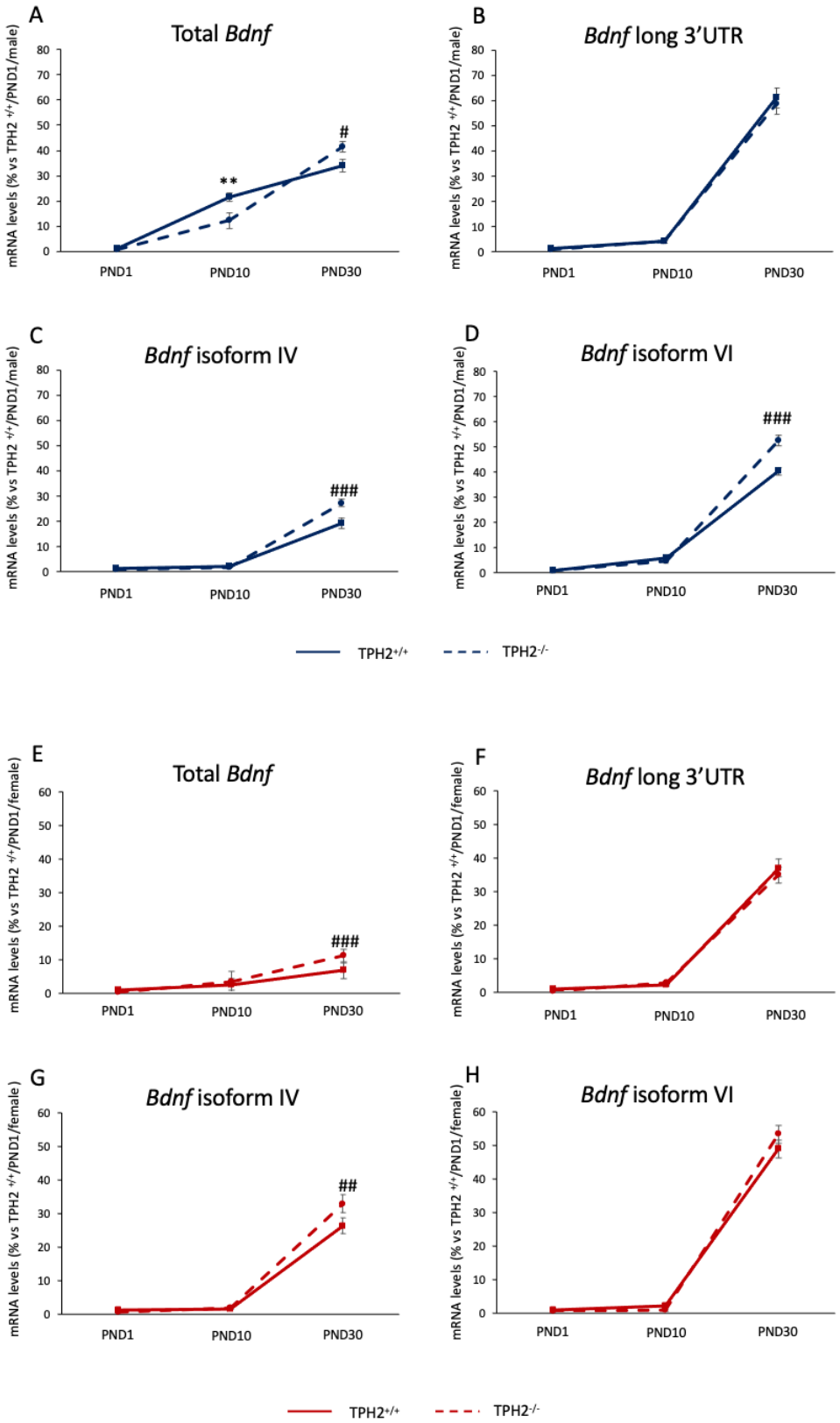

Figure 19: analyses of total *Bdnf* mRNA levels (A, E), *Bdnf* long 3'UTR (B, F), *Bdnf* isoform IV (C, G) and VI (D, H) mRNA levels in the PFC of $TPH2^{+/+}$ and $TPH2^{-/-}$ male and female rats at PND1, PND10 and PND30. The data are presented as percent change of $TPH2^{+/+}$/male and are expressed as mean±standard error of the mean (SEM) of at least 5 independent determinations. **p<0.01 vs $TPH2^{+/+}$/PND10; ##p<0.01; ###p<0.001 vs $TPH2^{+/+}$/PND30; two-way ANOVA with Fisher's PLSD.

4.2.2.3. TPH2$^{-/-}$ male and female showed a blunted transcription of the immediate early genes expression after stress exposure

In order to evaluate if the lack of serotonin influences the response to an acute stress challenge, we exposed the animals to 1 h of restraint stress and sacrificed them immediately at the end of the stress session (stress0') or 1 h later (stress60'). Since the implication of the immediate early genes in the response to acute stress (Molteni et al., 2009a) and their modulation due to the deletion of the SERT (Molteni et al., 2010) has been previously demonstrated, here, we measured two markers of neuronal activation, *Arc* and Fos Proto-Oncogene (*cFos*), to evaluate if the lack of serotonin in the CNS influenced the response to the restraint stress.

As shown in Figure 20A, *Arc* gene expression was significantly modulated by the stress ($F_{(2–101)}$ = 5.692, p<0.01; three-way ANOVA) with a genotype X stress interaction ($F_{(2–101)}$ = 7.181, p<0.01; three-way ANOVA). Accordingly, *Arc* mRNA levels were increased by stress exposure only in the TPH2$^{+/+}$ rats of both sexes (TPH2$^{+/+}$/stress60'/male: +97%, p<0.001 vs TPH2$^{+/+}$/naïve/male; TPH2$^{+/+}$/stress60'/male: +38%, p<0.05 vs TPH2$^{+/+}$/stress0'/male; TPH2$^{+/+}$/stress0'/female: +54%, p<0.01 vs TPH2$^{+/+}$/naïve/female; TPH2$^{+/+}$/stress60'/female: +42%, p<0.01 vs TPH2$^{+/+}$/naïve/female; Fisher's PLSD). Interestingly, this stress-mediated upregulation of *Arc* expression was completely blunted in TPH2$^{-/-}$ rats. Similarly, *cFos* expression was modulated by the stress ($F_{(2–90)}$ = 20.596, p<0.001; three-way ANOVA) with a genotype X stress interaction ($F_{(2–90)}$ = 19.110, p<0.001; three-way ANOVA). In line, we found an increase in *cFos* mRNA levels in TPH2$^{+/+}$ male rats at both time points after stress in comparison to naïve rats (TPH2$^{+/+}$/stress0'/male: +662%, p<0.001 vs TPH2$^{+/+}$/naïve/male; TPH2$^{+/+}$/stress60'/male: +336%, p < 0.01 vs TPH2$^{+/+}$/naïve/male; Fisher's PLSD). The upregulation of the *cFos* mRNA levels peaked at stress0' was markedly attenuated 1 h later (-43%, p<0.01 vs. TPH2$^{+/+}$/stress0'/male; Fisher's PLSD), confirming previously published data (Durchdewald et al., 2009). In female, acute challenge induced an upregulation of *cFos* mRNA levels in the TPH2$^{+/+}$/stress0' rats (+160%, p<0.001 vs TPH2$^{+/+}$/naïve/female; Fisher's PLSD) and stress60' group (+145%, p<0.01 vs TPH2$^{+/+}$/naïve/female; Fisher's PLSD). This characteristic pattern was again not observed in TPH2$^{-/-}$ female rats; moreover, 1 h after the stress exposure we found a significant downregulation of *cFos* mRNA levels in TPH2$^{-/-}$ rats (TPH2$^{-/-}$ /stress60'/female: -67%, p<0.01 vs TPH2$^{-/-}$/naïve/female; TPH2$^{-/-}$/stress60'/female: -65%, p<0.01 vs TPH2$^{-/-}$/ stress0'/female; Fisher's PLSD; Figure 20B).

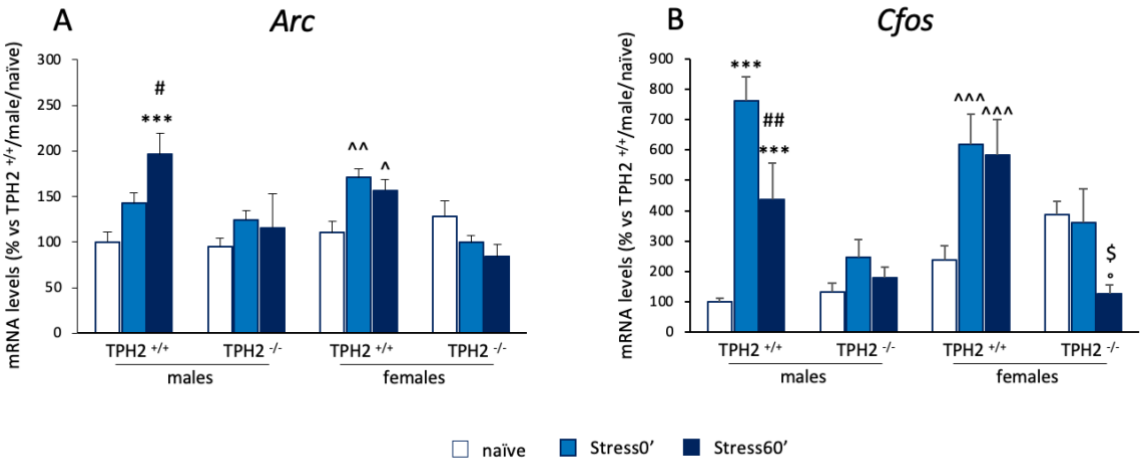

Figure 20: analyses of *Arc* (A) and *Cfos* (B) mRNA levels in the PFC of $TPH2^{+/+}$ and $TPH2^{-/-}$ male and female adult rats exposed to one single session of acute restraint stress and sacrificed after the stress or one hour later. The data are presented as percent change of $TPH2^{+/+}$/naïve/male and are expressed as mean±standard error of the mean (SEM) of at least 4 independent determinations. ***p<0.001 vs $TPH2^{+/+}$/naïve/male; #p<0.05; ##p<0.01 vs $TPH2^{+/+}$/stress0'/male; ^p<0.05; ^^p<0.01; ^^^p<0.001 vs $TPH2^{-/-}$/naïve/female; $p<0.05 vs $TPH2^{-/-}$/naïve/female; °p<0.05 vs $TPH2^{-/-}$/stress0'/female; three-way ANOVA with Fisher's PLSD.

4.2.2.4. Bdnf upregulation after acute stress exposure was partially impaired in TPH2$^{-/-}$ male and female rats

To evaluate the activation of Bdnf transcription after the stress, we measured the expression of total *Bdnf*, *Bdnf* long 3'UTR and *Bdnf* isoform IV and VI after in the animals exposed to the acute challenge.

As shown in Figure 21A, we found a significant effect of stress ($F_{(2–99)}$ = 112.032, p<0.001; three-way ANOVA) on the expression of the total form of the neurotrophin. Accordingly, total *Bdnf* mRNA levels were increased in both TPH2$^{+/+}$ and TPH2$^{-/-}$ male rats exposed to the acute stress and sacrificed at time point stress0' (TPH2$^{+/+}$/Stress0': +87%, p<0.001 vs TPH2$^{+/+}$/naïve/male; TPH2$^{-/-}$/Stress0': +51%, p<0.01 vs TPH2$^{-/-}$/naïve/male) and at time 60' (TPH2$^{+/+}$/stress60': +127%, p<0.001 vs TPH2$^{+/+}$/naïve/male; TPH2$^{-/-}$/Stress60': +82%, p<0.001 vs TPH2$^{-/-}$/naïve/male; Fisher's PLSD). In female rats, the exposure to stress increased the expression of total *Bdnf*, at both time points, in TPH2$^{+/+}$ (TPH2$^{+/+}$/stress0': +146%, p<0.001 vs TPH2$^{+/+}$/naïve/female; TPH2$^{+/+}$/stress60': +203%, p<0.001 vs TPH2$^{+/+}$/naïve/female; Fisher's PLSD) as well as in TPH2$^{-/-}$ (TPH2$^{-/-}$/stress0': +51%, p<0.01 vs TPH2$^{-/-}$/naïve/female; TPH2$^{-/-}$ /stress60': +88%, p<0.001 vs TPH2$^{-/-}$/naïve/female; Fisher's PLSD). Moreover, TPH2$^{+/+}$ stressed rats sacrificed 1 h after the stress exposure had higher mRNA levels compared to the stress0' groups (TPH2$^{+/+}$/stress60': +23%, p<0.01 vs TPH2$^{+/+}$/stress0'/females). Furthermore, the significant genotype X stress interaction ($F_{(2–98)}$ = 6.490, p<0.01) indicates that the upregulation found in TPH2$^{+/+}$ was higher than the one observed in TPH2$^{-/-}$ rats.

Bdnf long 3'UTR mRNA levels (Figure 21B) were significantly modulated by genotype X stress X sex interaction ($F_{(2–101)}$ = 3.203, p<0.05; three-way ANOVA). Moreover, we found a significant genotype X sex interaction ($F_{(2–101)}$ = 7.205, p<0.01; three-way ANOVA) reflecting an upregulation of *Bdnf* long 3'UTR expression in naïve female TPH2$^{+/+}$ compared to the male counterpart (TPH2$^{+/+}$/naïve/female: +30%, p<0.05 vs TPH2$^{+/+}$/naïve/male; Fisher's PLSD). Moreover, the significant effect of stress ($F_{(2–101)}$ = 109.926, p<0.001; three-way ANOVA) indicated that *Bdnf* long 3'UTR was increased after the acute challenge in male (TPH2$^{+/+}$/stress0'/male: +112%, p<0.001 vs TPH2$^{+/+}$/naïve/male; TPH2$^{+/+}$/stress60'/male: +161%, p<0.001 vs TPH2$^{+/+}$/naïve/male; TPH2$^{-/-}$/stress0'/male: +46%, p<0.01 vs TPH2$^{-/-}$ /naïve/male; TPH2$^{-/-}$/stress60'/male: +165%, p<0.001 vs. TPH2$^{-/-}$/naïve/male) and in female (TPH2$^{+/+}$/stress0'/female: +73%, p<0.001 vs TPH2$^{+/+}$/naïve/female; TPH2$^{+/+}$/stress60'/female: +88%, p<0.001 vs TPH2$^{+/+}$/naïve/female; TPH2$^{-/-}$/stress0'/female: +31%, p<0.05 vs TPH2$^{-/-}$

/naïve/female; TPH2$^{-/-}$/stress60'/female: +46%, p<0.001 vs TPH2$^{-/-}$/naïve/female), independently from the genotype. Moreover, in male rats the up-regulation observed 1 h after the stress exposure was significantly higher than the increase found at time 0' in both genotype (TPH2$^{+/+}$/stress60'/male: +23%, p<0.001 vs TPH2$^{+/+}$/stress0'/male; TPH2$^{-/-}$ /stress60'/male: +81%, p<0.001 vs TPH2$^{-/-}$/stress0'/male). Finally, the significant genotype X stress interaction ($F_{(2–101)}$ = 4,368, p<0.05; three-way ANOVA) indicates that the increase in expression of *Bdnf* long 3'UTR due to stress exposure was more robust in TPH2$^{+/+}$ than in TPH2$^{-/-}$ rats.

Analysis of *Bdnf* isoform IV mRNA levels (Figure 21C) revealed a significant effect of stress ($F_{(2–101)}$ = 66.292, p<0.001; three-way ANOVA). In male, *Bdnf* isoform IV expression was upregulated by stress in TPH2$^{+/+}$ rats (TPH2$^{+/+}$/stress0'/male: +88%, p<0.001 vs TPH2$^{+/+}$/naïve/male; TPH2$^{+/+}$ /stress60'/male: +141%, p<0.001 vs TPH2$^{+/+}$/naïve/male) with a further increase from stress point 0' to 60' (TPH2$^{+/+}$/stress60'/male: +28%, p<0.05 vs TPH2$^{+/+}$/stress0'/male) and in TPH2$^{-/-}$ (TPH2$^{-/-}$/stress0'/male: +37%, p<0.01 vs TPH2$^{-/-}$ /naïve/male; TPH2$^{-/-}$/stress60'/male: +41%, p<0.01 vs TPH2$^{-/-}$/naïve/male). Moreover, *Bdnf* IV was increased in unstressed TPH2$^{-/-}$ in comparison to TPH2$^{+/+}$ rats (TPH2$^{-/-}$/naïve/male: +39%, p<0.01 vs TPH2$^{+/+}$/naïve/male). In female, exposure to the acute stress increased the *Bdnf* isoform IV mRNA levels in both genotypes (TPH2$^{+/+}$/stress0'/female: +98%, p<0.001 vs TPH2$^{+/+}$/naïve/female; TPH2$^{+/+}$/stress60'/female: +152%, p<0.001 vs TPH2$^{+/+}$/naïve/female; TPH2$^{+/+}$/stress60'/female: +27%, p<0.05 vs TPH2$^{+/+}$/stress0'/female; TPH2$^{-/-}$/stress0'/female: +39%, p<0.05 vs TPH2$^{-/-}$/naïve/female; TPH2$^{-/-}$/stress60'/female: +46%, p<0.01 vs TPH2$^{-/-}$ /naïve/female). Similar to total *Bdnf* and *Bdnf* long 3'UTR, the significant genotype X stress interaction ($F_{(2–101)}$ = 11.065, p<0.001; three-way ANOVA) was also observed for the *Bdnf* isoform IV, suggesting a different response to the challenging situation in the two genotypes in terms of magnitude of the effect.

Finally, *Bdnf* isoform VI mRNA levels (Figure 21D) were affected by the genotype ($F_{(2–100)}$ = 10.338, p<0.01; three-way ANOVA) reflecting the slightly higher mRNA levels found in unstressed male TPH2$^{-/-}$ compared to unstressed TPH2$^{-/-}$male rats (TPH2$^{-/-}$/naïve/male: +22%, p<0.05 vs TPH2$^{-/-}$/naïve/male). Moreover, in females, isoform VI expression was downregulated specifically in TPH2$^{+/+}$ rats (TPH2$^{+/+}$/stress0'/female: -32%, p<0.05 vs TPH2$^{+/+}$/naïve/female; TPH2$^{+/+}$/stress60'/female: -26%, p<0.05 vs TPH2$^{+/+}$/naïve/female), while its levels were increased in TPH2$^{-/-}$ stressed rats sacrificed 1 h after the stress exposure

in comparison to the animals of the same genotype killed immediately after the acute stress (TPH2$^{+/+}$/stress60'/female: +45%, p<0.05 vs TPH2$^{+/+}$/stress0'/female).

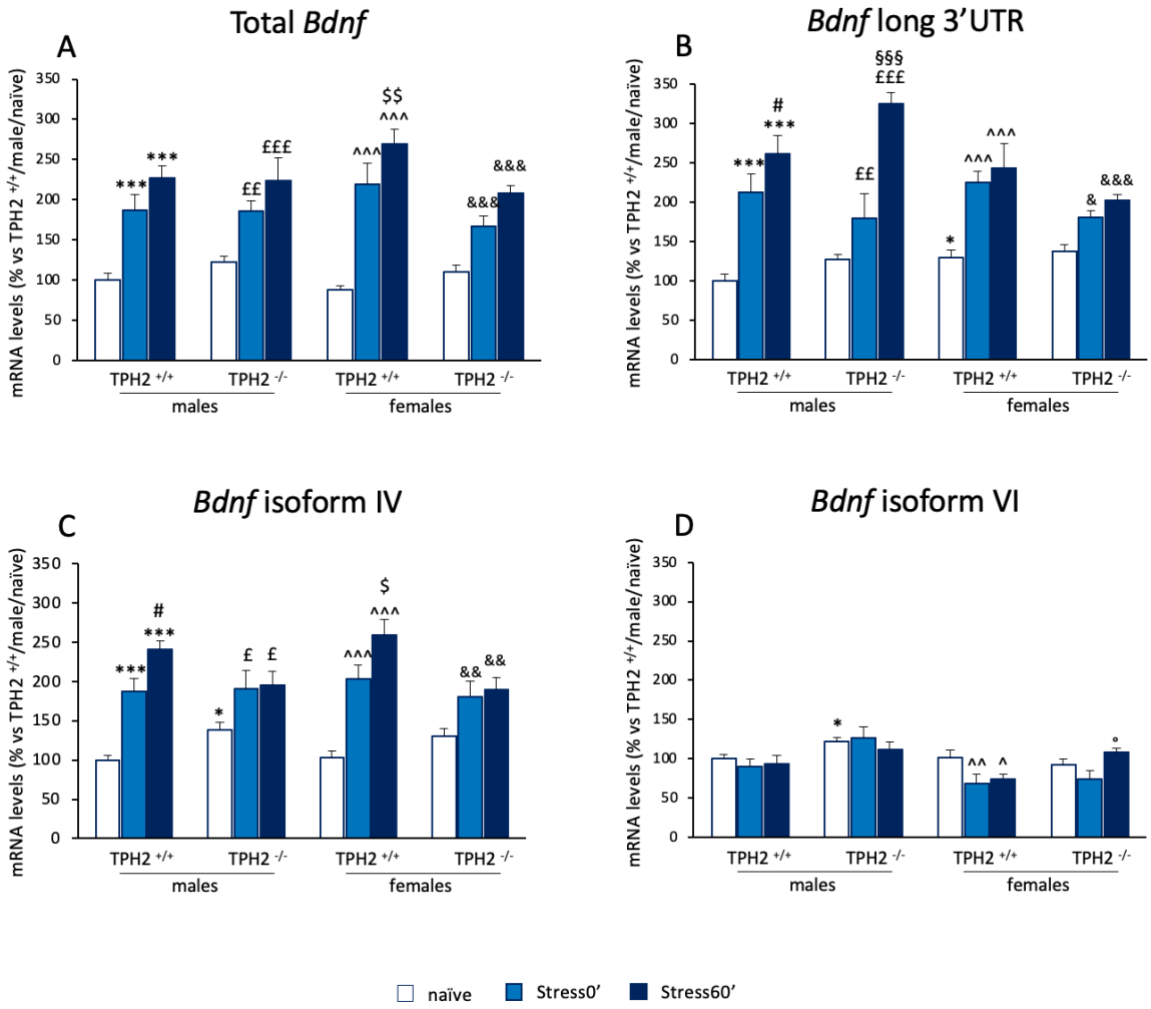

Figure 21: analyses of total *Bdnf* (A), *Bdn*f long 3'UTR (B), *Bdnf* isoform IV (C) and VI (D) mRNA levels in the PFC of TPH2$^{+/+}$ and TPH2$^{-/-}$ male and female adult rats exposed to one single session of acute restraint stress and sacrificed after the stress or one hour later. The data are presented as percent change of TPH2$^{+/+}$/naïve/male and are expressed as mean±standard error of the mean (SEM) of at least 4 independent determinations. *p<0.05; ***p<0.001 vs TPH2$^{+/+}$/naïve/male; #p<0.05 vs TPH2$^{+/+}$/stress0'/male; £p<0.05; ££p<0.01; £££p<0.001 vs TPH2$^{-/-}$/naïve/male; §§§p<0.001 vs TPH2$^{-/-}$/stress 0'/male; ^p<0.05; ^^p<0.01; ^^^p<0.001 vs TPH2$^{+/+}$/naïve/female; $p<0.05; $$p<0.01 vs TPH2$^{+/+}$/stress 0'/female; &p<0.05; &&p<0.01; &&&p<0.001 vs TPH2$^{-/-}$/naïve/female; °p<0.05 vs TPH2$^{-/-}$/stress0'/female; three-way ANOVA with Fisher's PLSD.

4.2.2.5. Corticosterone release after stress exposure was not altered by the absence of central serotonin

After stressful events, another system rapidly activated is the HPA axis activation which results in the release of corticosterone from the adrenal glands (McEwen, 2007). Here, we evaluated if the lack of central serotonin could interfere with the release of this hormone in the bloodstream.

In male rats, plasma corticosterone levels were significantly modulated by the acute stress ($F_{(1–27)}$ = 60.729, p<0.001, two-way ANOVA). Indeed, we found an increase in corticosterone levels in rats of both genotypes at stress0' time point in comparison to naïve rats ($TPH2^{+/+}$/stress0': +99%, p<0.001 vs $TPH2^{+/+}$/naïve; $TPH2^{-/-}$/stress0': +98%, p<0.001 vs. $TPH2^{-/-}$/naïve; Fisher's PLSD), while a significant decrease was observed in the group of rats killed at stress60' time point compared to the control group of the same genotype ($TPH2^{+/+}$/stress60': -61%, p<0.01 vs $TPH2^{+/+}$/ naïve; $TPH2^{-/-}$/stress60': -54%, p<0.05 vs $TPH2^{-/-}$/naïve; Fisher's PLSD) as well as to stress0' groups ($TPH2^{+/+}$/stress60': -81%, p<0.001 vs $TPH2^{+/+}$/stress0'; $TPH2^{-/-}$/stress60': -77%, p < 0.001 vs $TPH2^{-/-}$/stress0'; Fisher's PLSD; Figure 22A). As shown in Figure 22B, female rats showed a higher heterogeneity in corticosterone levels at basal level and in response to stress. Statistical analysis revealed significant modulation of plasma corticosterone levels by acute stress only in $TPH2^{-/-}$ rats ($F_{(1–42)}$ = 5.239, p<0.05; two-way ANOVA), with an upregulation of corticosterone levels in stress0' group compared to naïve rats (+82%, p<0.05 vs $TPH2^{-/-}$/ naïve; Fisher's PLSD) and a decrease at stress60' time point with respect to $TPH2^{-/-}$/stress0' (-50%, p<0.05 vs $TPH2^{-/-}$/stress0'; Fisher's PLSD).

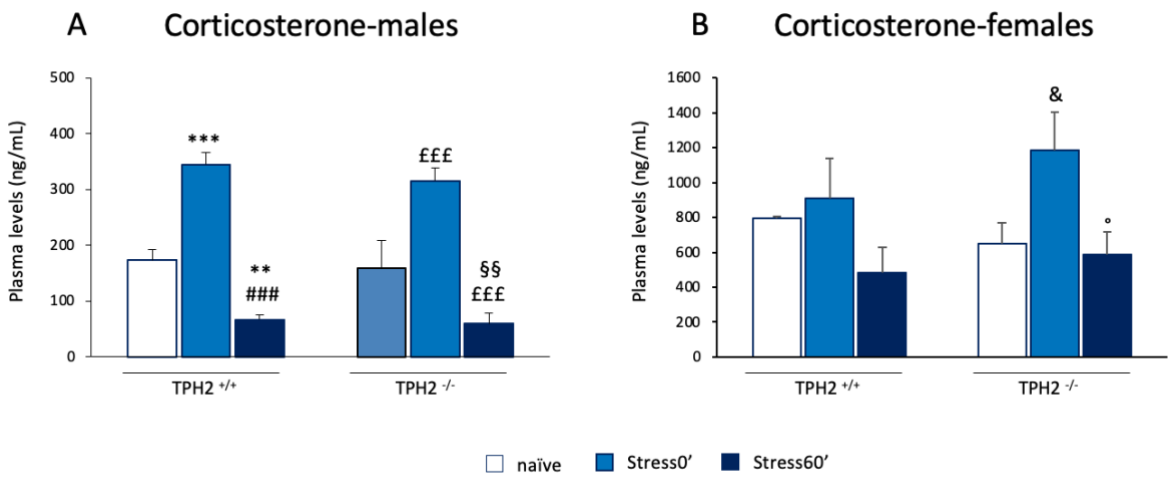

Figure 22: analyses of corticosterone levels in the blood stream of $TPH2^{+/+}$ and $TPH2^{-/-}$ male (A) and female (B) adult rats exposed to one single session of acute restraint stress and sacrificed after the stress or one hour later. The data are expressed as mean±standard error of the mean (SEM) of at least 4 independent determinations. **p<0.01; ***p<0.001 vs $TPH2^{+/+}$/naïve/male; ###p<0.001 vs $TPH2^{+/+}$/stress0'/male; £££ p<0.001 vs $TPH2^{-/-}$/naïve/male; §§p<0.01 vs $TPH2^{-/-}$/stress 0'/male; &p<0.05 vs $TPH2^{-/-}$/naïve/female; °p<0.05 vs $TPH2^{-/-}$/stress 0'/female; two-way ANOVA with Fisher's PLSD.

4.2.2.6. Glucocorticoid receptor translocation into the nucleus induced by acute stress exposure is blunted in TPH2$^{-/-}$ rats

As observed before, the activation of the HPA axis after acute restraint stress results in the release of corticosterone from the adrenal glands and subsequent response in the extrinsic HPA axis structures, such as PFC via activation of the genomic pathway of the GRs (Adzic et al., 2009). In particular, the binding of the hormone to its receptor induces its translocation into the nucleus (Revollo and Cidlowski, 2009). Hence, to analyze the responsiveness of TPH2$^{+/+}$ and TPH2$^{-/-}$ rats in terms of the activation of the genomic pathway of the glucocorticoids, we first analyzed the GR protein levels in the nuclear and cytosolic fractions immediately after the end of the acute stress, the time point where we observed the increased release of the hormone in the bloodstream. Moreover, seen the extreme heterogeneity of female rats in terms of corticosterone levels, we focused specifically on male animals. As shown in Figure 23A, E, we found a significant genotype X stress interaction ($F_{(1,16)}$ = 4.971 $p<0.05$; two-way ANOVA) in GR protein levels in the nuclear compartment, but no effects of the genotype ($F_{(1,16)}$ = 3.055 $p>0.05$; two-way ANOVA) and of the stress ($F_{(1,16)}$ = 3.747 $p>0.05$; two-way ANOVA). Indeed, the receptor was significantly up-regulated by the stress in the nuclear compartment of TPH2$^{+/+}$ rats (+89% $p<0.05$ vs. TPH2$^{+/+}$/naïve; Fisher's PLSD), but not in the TPH2$^{-/-}$ counterpart. On the contrary, we did not find any significant modulation of GR protein levels in the whole homogenate and the cytosolic compartment even if a slight decrease of its levels was observed in the cytosol of TPH2$^{+/+}$ stressed rats (-27% $p>0.05$ vs TPH2$^{+/+}$/naïve; Fisher's PLSD) (Figure 23B, C, E). Finally, we looked at the ratio of the nuclear vs cytosolic GR levels as an indicator of the translocation into the nucleus of the receptor after stress exposure and we found a borderline significance for the genotype X stress interaction ($F_{(1,13)}$ = 4.761 $p=0.054$; two-way ANOVA). Indeed, following the results described in panels A and B, we observed an increased translocation in TPH2$^{+/+}$ stressed animals (+191% $p<0.05$ vs TPH2$^{+/+}$/naïve; Fisher's PLSD) while this effect was blunted in TPH2$^{-/-}$ rats (Figure 23D).

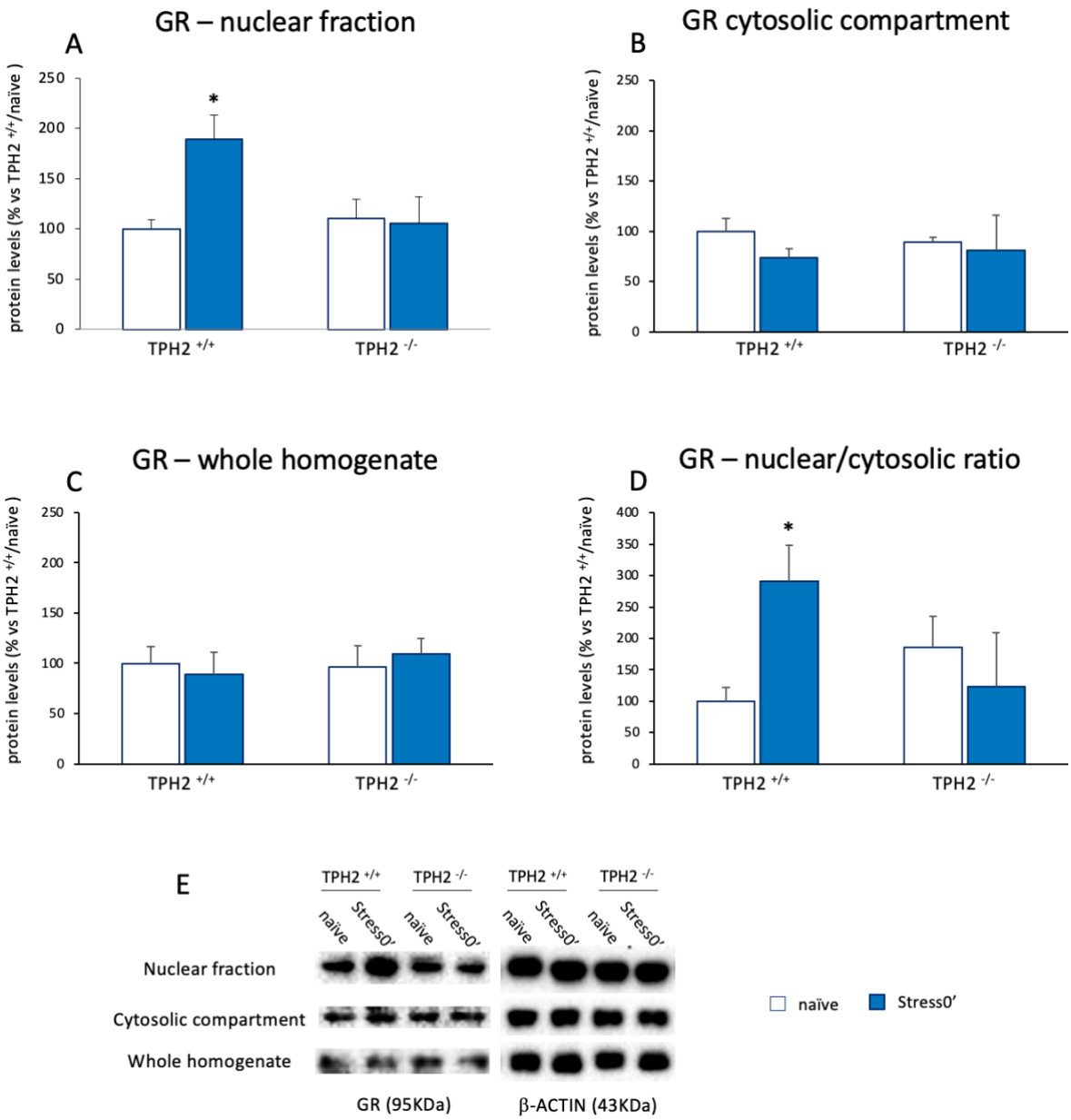

Figure 23: analyses of GR protein levels in the nuclear fraction (A), cytosolic compartment (B), whole homogenate (C) and the ratio between the nuclear and the cytosolic compartment (D) in the PFC of TPH2$^{+/+}$ and TPH2$^{-/-}$ male adult rats exposed to one single session of acute restraint stress and sacrificed after the stress or one hour later. Panel E is a representative of western-blot bands of GR and β-ACTIN used as internal control. The data are presented as percent change of TPH2$^{+/+}$/naïve/ and are expressed as mean±standard error of the mean (SEM) of at least 3 independent determinations. *p<0.05; vs TPH2$^{+/+}$/naïve; two-way ANOVA with Fisher's PLSD.

4.2.2.7. MAZ1 transcription factor is increased after stress exposure in TPH2$^{+/+}$ but not in TPH2$^{-/-}$ stressed rats

Once in the nucleus, GRs bind the GREs, and this binding is facilitated by the presence of cofactors such as Myc-associated zinc finger protein 1 (MAZ1) and Specificity Protein 1 (SP1) (Datson et al., 2011). In the whole homogenate, MAZ1 protein levels showed a significant genotype X stress interaction ($F_{(1,15)}$ = 6.424 p<0.05; two-way ANOVA) but no effect of the genotype ($F_{(1,15)}$ = 3.216 p>0.05; two-way ANOVA) and of the stress ($F_{(1,15)}$ = 0.029 p>0.05; two-way ANOVA). Accordingly, we observed a significant increase of MAZ1 protein levels in TPH2$^{+/+}$ rats exposed to stress when compared to TPH2$^{-/-}$stressed animals (+121%, p<0.01 vs TPH2$^{-/-}$ /stress; Fisher's PLSD) (Figure 24A, C). We did not find any changes in SP1 protein levels (Figure 24B, C).

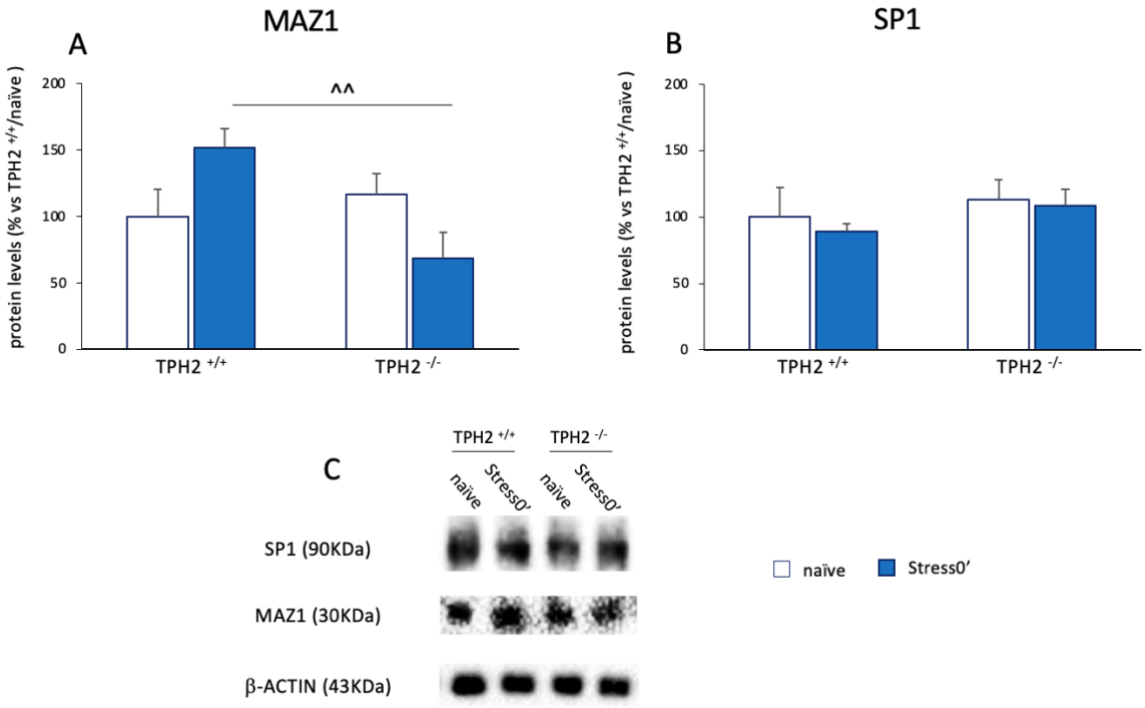

Figure 24: analyses of MAZ1, SP1 protein levels in the whole homogenate of the PFC of TPH2$^{+/+}$ and TPH2$^{-/-}$ male and female adult rats exposed to one single session of acute restraint stress and sacrificed after the stress or one hour later. Panel E is a representative of western-blot bands of MAZ1, SP1 and β-ACTIN used as internal control. The data are presented as percent change of TPH2$^{+/+}$/naïve/ and are expressed as mean±standard error of the mean (SEM) of at least 3 independent determinations. ^^p<0.01; vs TPH2$^{+/+}$/Stress0'; two-way ANOVA with Fisher's PLSD.

4.2.2.8. The up-regulation of glucocorticoid responsive gene expression is blunted in TPH2$^{-/-}$ stressed rats

The translocation into the nucleus of the GRs leads to the expression of the glucocorticoid responsive genes, carrying the GREs. As shown in Figure 25A, Dual Specificity Phosphatase 1 (*Dusp1*) mRNA levels were significantly affected by the stress ($F_{(1,33)}$ = 10.770 p<0.01, two-way ANOVA), that induced an increase in the expression of this gene specifically in TPH2$^{+/+}$ rats (+53%, p<0.01 vs TPH2$^{+/+}$/naïve; Fisher's PLSD). Moreover, Growth Arrest And DNA Damage Inducible β (*Gadd45*β) expression (Figure 25B) was significantly affected both by the genotype and by the stress (genotype: $F_{(1,34)}$ = 8.765 p<0.05 and stress: $F_{(1,34)}$ = 85.447 p<0.001; two-way ANOVA). Indeed, stress exposure induced an increased transcription not only in TPH2$^{+/+}$ rats (+113% p<0.001 vs TPH2$^{+/+}$/naïve; Fisher's PLSD) but also in TPH2$^{-/-}$ animals (+40%, p<0.01 vs TPH2$^{-/-}$/naïve; Fisher's PLSD). However, as demonstrated by the significant genotype X stress interaction ($F_{(1,34)}$ = 16.447 p<0.001; two-way ANOVA), the up-regulation due to the challenge found in TPH2$^{+/+}$ was more robust (+39%, p<0.001 vs TPH2$^{-/-}$/stress; Fisher's PLSD) compared to those found in TPH2$^{-/-}$ stressed rats. Similarly, Nuclear Receptor Subfamily four Group A Member 1 (*Nr4a1*) was affected by the genotype ($F_{(1,34)}$ = 4.602 p<0.05, two-way ANOVA) and by the stress ($F_{(1,34)}$ = 101.764 p<0.001, two-way ANOVA) with a significant interaction between the two variables ($F_{(1,34)}$ = 16.650 p<0.001, two-way ANOVA). Accordingly, *Nr4a1* expression was up-regulated both in TPH2$^{+/+}$ (+153% vs. TPH2$^{+/+}$/naïve; Fisher's PLSD) and in TPH2$^{-/-}$ (+54% vs TPH2$^{-/-}$/naïve; Fisher's PLSD) in comparison to their controls. Again, we observed a higher expression of *Nr4a1* in TPH2$^{+/+}$ in comparison to TPH2$^{-/-}$stressed rats (+36% p<0.001 vs. TPH2$^{-/-}$/stress; Fisher's PLSD) (Figure 25C).

On the contrary, Serum/Glucocorticoid Regulated Kinase (*Sgk1*), TSC22 Domain Family Member 3 (*Gilz*) and FKBP Prolyl Isomerase 5 (*Fkbp5*) mRNA levels were affected only by the stress (*Sgk1*: $F_{(1,35)}$ = 135.512 p<0.001, two-way ANOVA; *Gilz*: $F_{(1,35)}$ = 96.360 p<0.001, two-way ANOVA; *Fkbp5*: $F_{(1,33)}$ = 16.167 p<0.001, two-way ANOVA). In particular, we observed a comparable increase of the expression of these genes after the acute challenge both in TPH2$^{+/+}$ (*Sgk1*: +102% p<0.001; *Gilz*: +130% p<0.001; *Fkbp5*: +39% p<0.01 vs TPH2$^{+/+}$/naïve) and TPH2$^{-/-}$ rats (*Sgk1*: +117% p<0.001; *Gilz*: +107% p<0.001; *Fkbp5*: +25% p<0.05 vs TPH2$^{-/-}$ /naïve; Fisher's PLSD) (Figures 25D, E, F).

Also, S100A10 (*P11*) mRNA levels were affected by the stress ($F_{(1,35)}$ = 6.158 p<0.05, two-way ANOVA). However, stress exposure induced a reduction in *P11* expression in both genotypes

after stress exposure ($TPH2^{+/+}$: -21% p>0.05 vs $TPH2^{+/+}$/naïve; $TPH2^{-/-}$: -28% p<0.05 vs $TPH2^{-/-}$/naïve; Fisher's PLSD). Moreover, $TPH2^{-/-}$ rats showed increased levels of *P11* mRNA when compared to $TPH2^{+/+}$ (+43% p<0.05 vs $TPH2^{+/+}$/naïve; Fisher's PLSD) (Figure 25G).

Finally, as shown in Figure 25H, we did not find any changes in Forkhead box O1 (*FoxO1*) expression.

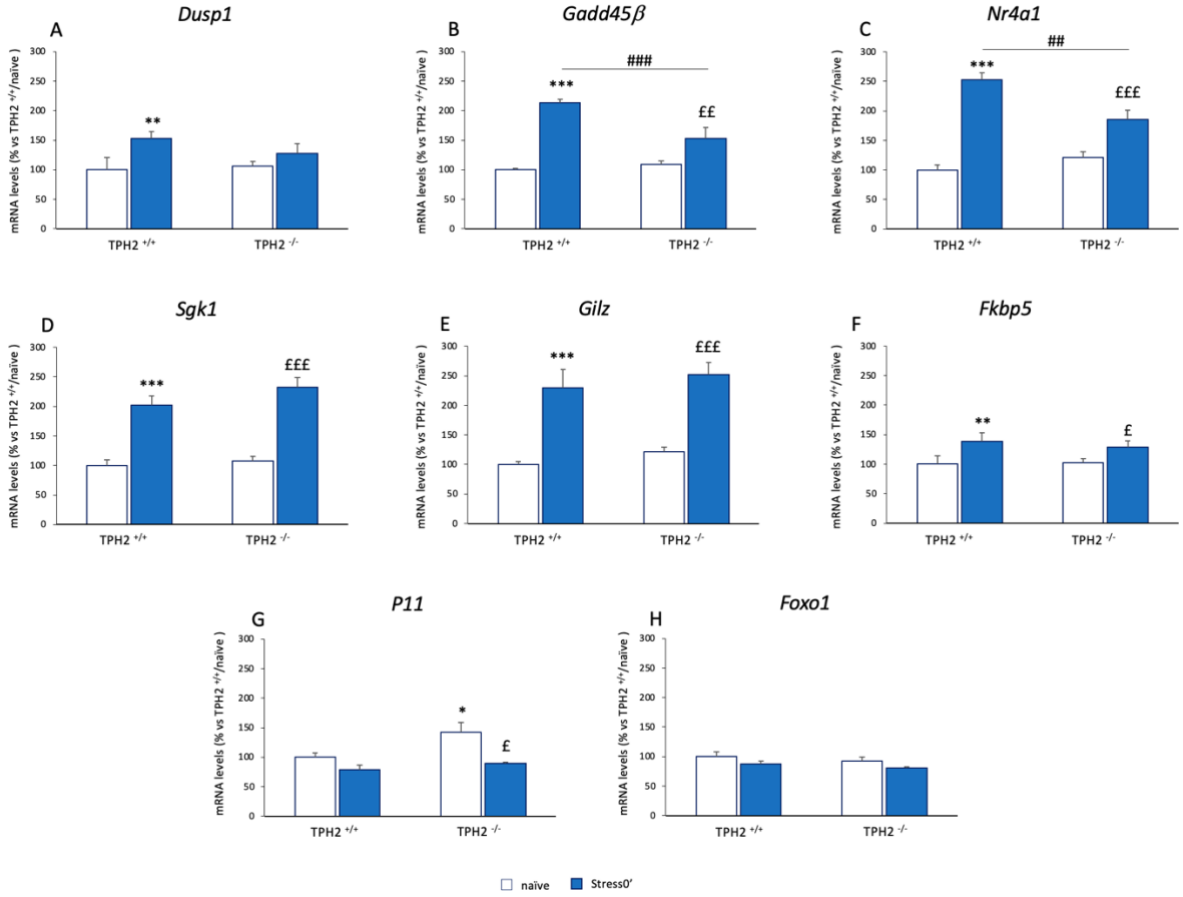

Figure 25: analyses of *Dusp1* (A), *Gadd45b* (B), *Nr4a1* (C), *Sgk1* (D), *Gilz* (E), *Fkbp5* (F), *P11* (G) and *Foxo1* (H), mRNA levels in the PFC of $TPH2^{+/+}$ and $TPH2^{-/-}$ male and female adult rats exposed to one single session of acute restraint stress and sacrificed after the stress. The data are presented as percent change of $TPH2^{+/+}$/naïve/male and are expressed as mean±standard error of the mean (SEM) of at least 4 independent determinations. *p<0.05; **p<0.01; ***p<0.001 vs $TPH2^{+/+}$/naïve; ##p<0.01; ###p<0.001 vs $TPH2^{+/+}$/stress0'; £p<0.05; ££p<0.01; £££p<0.001 vs $TPH2^{-/-}$/naïve; two-way ANOVA with Fisher's PLSD.

4.2.2.9. Serotonin Deficiency Alters Clock Gene Expression Profile

It has been demonstrated that acute stress exposure can influence the expression of period circadian regulator 1 (*Per1*) thanks to the presence of a canonical GRE (Yamamoto et al., 2005). Hence, seen the role of *Per1* in the control of circadian rhythms, we measured the expression of the main components of the clock machinery. In our animals, we found a significant genotype X stress interaction ($F_{(1,35)}$ = 7.098 p<0.05, two-way ANOVA) and a significant effect of the genotype ($F_{(1,35)}$ = 15.130 p<0.01, two-way ANOVA) and of the stress ($F_{(1,35)}$ = 115.534 p<0.001, two-way ANOVA) on *Per1* expression. Indeed, as shown in Figure 26A, *Per1* mRNA levels were significantly up-regulated in $TPH2^{+/+}$ stressed rats in comparison to their naïve controls (+80% p<0.001 vs $TPH2^{+/+}$/naïve; Fisher's PLSD). This increase was present also in $TPH2^{-/-}$ rats exposed to the restraint stress (+52% p<0.001 vs $TPH2^{-/-}$/naïve; Fisher's PLSD) even if the effect was more pronounced in $TPH2^{+/+}$ animals (+28% p<0.01 vs $TPH2^{+/+}$/stress; Fisher's PLSD).

Also, the expression of period circadian regulator 2 (*Per2*) showed a significant genotype X stress interaction ($F_{(1,34)}$ = 5.725 p<0.05, two-way ANOVA) and a significant effect of the stress ($F_{(1,34)}$ = 55.671 p<0.001, two-way ANOVA). However, *Per2* expression was increased more in $TPH2^{-/-}$ stressed animals than in $TPH2^{+/+}$ ($TPH2^{+/+}$: +37% p<0.01 vs $TPH2^{+/+}$/stress; $TPH2^{-/-}$: +75% p<0.001; Fisher's PLSD). Indeed, the increased transcription of *Per2* due to stress exposure was higher in $TPH2^{-/-}$compared to $TPH2^{+/+}$ animals (+24% p<0.05 vs $TPH2^{+/+}$/stress; Fisher's PLSD) (Figure 26B). On the contrary, we did not find any changes in cryptochrome circadian regulator 1 (*Cry1*) and 2 (*Cry2*) expression (Figures 26C, D).

Similar to what was observed in *Per1* expression, *Reverb-*α mRNA levels were affected by the genotype and by the stress with a significant genotype X stress interaction (genotype: $F_{(1,35)}$ = 10.544 p<0.05; stress: $F_{(1,35)}$ = 6.112; genotype X stress interaction: $F_{(1,35)}$ = 4.984 p<0.05; p<0.05 two-way ANOVA). Specifically, *Rev-erb*α expression was increased in $TPH2^{+/+}$ stressed animals (+31% p<0.01 vs $TPH2^{+/+}$/naïve; Fisher's PLSD) while we did not observe any changes in $TPH2^{-/-}$rats (Figure 26E).

Finally, as shown in Figure 26F, nuclear receptor subfamily 1, group D member 1 (*Rev-erb*β) expression showed a significant genotype X stress interaction ($F_{(1,34)}$ = 4.789 p<0.05 two-way ANOVA). Indeed, we observed an up-regulation in *Rev-erb*β expression in $TPH2^{-/-}$in comparison to $TPH2^{+/+}$ counterpart (+20% p<0.01 vs $TPH2^{+/+}$/naïve; Fisher's PLSD). Moreover, nuclear receptor subfamily 1, group D

member 2 (*Rev-erbβ*) levels were normalized in TPH2$^{-/-}$ rats after the stress (-22% p<0.01 vs TPH2$^{-/-}$/naïve; Fisher's PLSD).

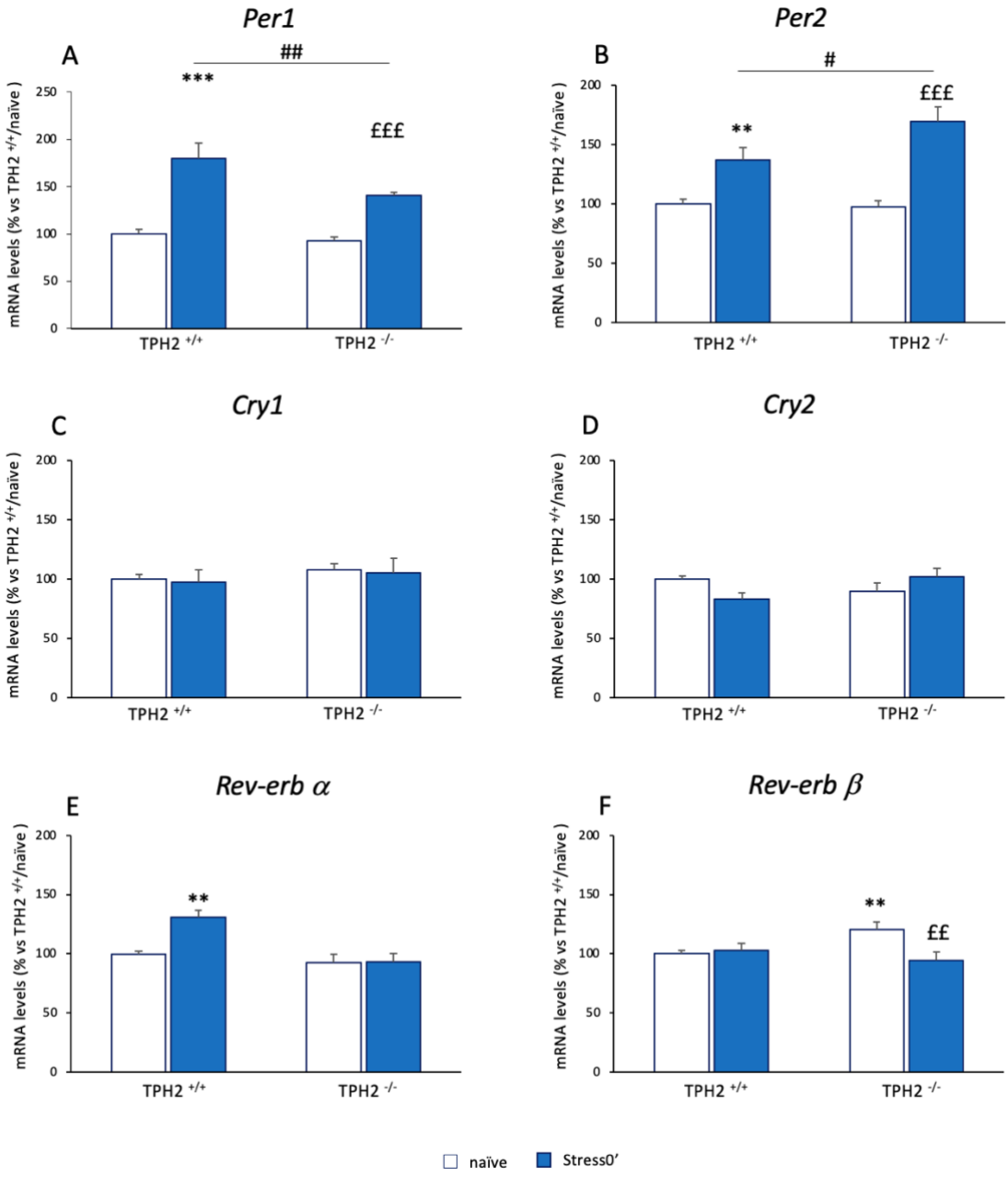

Figure 26: analyses of *Per1* (A), *Per2* (B), *Cry1* (C), *Cry2* (D), *Rev-erb α* (E), and *Rev-erb β* (F), mRNA levels in the PFC of TPH2$^{+/+}$ and TPH2$^{-/-}$ male and female adult rats exposed to one single session of acute restraint stress and sacrificed after the stress. The data are presented as percent change of TPH2$^{+/+}$/naïve/male and are expressed as mean±standard error of the mean (SEM) of at least 4 independent determinations. **p<0.01; ***p<0.001 vs TPH2$^{+/+}$/naïve; #p<0.05; ##p<0.01; vs TPH2$^{+/+}$/stress0'; ££p<0.01; £££p<0.001 vs TPH2$^{-/-}$/naïve; two-way ANOVA with Fisher's PLSD.

4.2.3. Discussion

The results of the present study highlight that the deletion of TPH2 profoundly affects neuroplastic mechanisms from the first stages of life until adulthood and influences the response to an acute stress both interfering with the activation of Bdnf machinery as well as with the genomic pathway of the glucocorticoid receptor. Moreover, serotonin deficiency into the brain modulates the transcription of clock genes suggesting a possible alteration in circadian rhythms under stress conditions.

At basal level, we found that the lack of central serotonin modulated the neuroplastic mechanisms by increasing the expression of *Bdnf* both in male and females TPH2$^{-/-}$ adult rats. Indeed, total *Bdnf* expression was up-regulated in a condition of absence of the neurotransmitter in the brain. Interestingly, the contribution of different *Bdnf* isoforms to this increase is sex specific: in male TPH2$^{-/-}$ rats, it was due to enhancement of all *Bdnf* transcripts investigated, while in females only the isoform IV was upregulated. These results are in line with our previous data obtained in SERT$^{-/-}$ rats (Molteni et al., 2010; Calabrese et al., 2013): while hyperserotonergia induced by SERT deletion impairs neuronal plasticity in the PFC and hippocampus by downregulating Bdnf (Homberg et al., 2007; Olivier et al., 2008), serotonin depletion in TPH2$^{-/-}$ resulted in an opposite effect. These effects in the transcription were mirrored, at translational level, by an upregulation of the mature form of BDNF protein levels in the crude synaptosomal fraction in both male and female TPH2$^{-/-}$ rats. The enhancement of the protective form of the neurotrophin, that regulates synaptic connections and plasticity (Martinowich et al., 2007) may be indicative of an increase in the pool of the neurotrophin ready for the release (Poo, 2001; Lau et al., 2010). This suggests a potential compensatory mechanism in the brain to deal with the absence of the trophic contribution of serotonin, confirming data previously obtained in PFC (Kronenberg et al., 2016) and hippocampus (Migliarini et al., 2013) of TPH2-deficient mice.

Furthermore, it's well known that ontogenesis is a critical period for brain adaptability and plasticity. Several lines of evidence support the fundamental role of serotonin during neurodevelopment (Lauder, 1993; Gaspar et al., 2003) as well as the crucial role of BDNF in promoting brain growth and in the establishment of neural circuitry including the serotoninergic system (Mamounas et al., 1995). Accordingly, we observed a stepwise increase in *Bdnf* expression from PND1 to PND30 in wild-type animals as previously demonstrated (Calabrese et al., 2013). Although a similar profile was observed in TPH2$^{-/-}$ rats, we highlighted

that the increased levels of Bdnf present at adulthood was already established in early adolescence with an increase of its transcription in TPH2$^{-/-}$ rats at PND30 in both sexes. In males, this effect was paralleled by an upregulation of *Bdnf* isoform IV and VI, while in females this effect was, again, restricted to the specific effect on the expression of the isoform IV.

Despite we did not find an overall modulation in *Bdnf* expression at early stages (PND1 and PND10), we observed a downregulation in the total *Bdnf* expression in TPH2$^{-/-}$ in comparison to TPH2$^{+/+}$ PND10 male rats. This suggests that peripheral sources may compensate for the lack of central serotonin synthesis during the early postnatal periods of life. Indeed, the blood-brain barrier is not fully functional before PND12 (Ribatti et al., 2006) and serotonin can reach the brain from the placenta during the embryonic stages (Cool et al., 1990; Carrasco et al., 2000). Similarly, serotonin produced in the periphery could easily enter the brain after birth until the complete maturation of the blood-brain barrier. Accordingly, Vitalis et al., (2007) demonstrated that the embryonic transient depletion of serotonin does not modify cortical BDNF levels until PND21 in pups. In summary, these results suggest that the activation of trophic mechanisms set in motion to compensate the serotonin synthesis deficiency at central level became more evident starting from PND30, when there is no more supply of peripheral serotonin sources, such as placenta and blood. Moreover, the different regulation of *Bdnf* isoforms we found in females in comparison to males may be due to the fact that different transcripts may have different subcellular localization (Chiaruttini et al., 2008) and are controlled by specific intracellular pathways (Aid et al., 2007). Among these, sex hormones are known to contribute to BDNF modulations (Chan and Ye, 2017), in line with the finding that estrogens can induce Bdnf expression by activating their receptors and may modify the neurotrophin activity through methylation of the *Bdnf* promoter IV and V in the hippocampus (Moreno-Piovano et al., 2014). Moreover, sex steroids can influence serotonergic neurotransmission (Dalla et al., 2005; Kokras et al., 2009). In line, it has been demonstrated that androgens facilitate serotonin binding to its transporter SERT, while estrogens seem to delay it (Kranz et al., 2015). However, due to the limited information available on the functional role of each *Bdnf* transcript, it is not feasible to draw clear-cut conclusions on the consequences exerted by differences in isoform expression between males and females.

Seeing that, in basal condition, the lack of central serotonin was in some way compensated by neuroplastic mechanisms and considering the well-established relationship between the serotonergic system and the stress reactivity (Chen and Miller, 2012; Homberg et al., 2014)

we then tested if the lack of central serotonin could affect the ability to cope with more dynamic conditions. Hence, we exposed adult rats to 1 h of restraint stress and we sacrificed them immediately at the end of the stress session or 1 h later. Since the implication of neuronal activation in response to acute stress can be evaluated by the expression of immediate early genes (Ons et al., 2004; Molteni et al., 2009a) and in order to assess a different outcome to the acute restraint stress exposure driven by serotonin deficiency, we measured two markers of neuronal activation, *Arc* and *cFos*. As expected, in both males and female wild-type rats we observed a clear stress response, with the slow increase in *Arc* expression during and 1 h after stress and a quick upregulation at stress0' and a downregulation 1 h later in *cFos* expression, confirming previously published data (Durchdewald et al., 2009). Interestingly in both males and females, the lack of central serotonin influenced the response to the challenging condition by preventing the upregulation of both the immediate early genes transcription found in $TPH2^{+/+}$ rats. Seen that *Arc* mediates the translation between neuronal activation-induced changes into sustained structural and functional modification at synaptic levels (Alberi et al., 2011), the lack of activation observed in $TPH2^{-/-}$ rats may reflect an incorrect translation of the stimulus into a more stable outcome. Moreover, we have previously shown that hyperserotonergia, evoked by the lack of SERT, has an opposite effect on the stress-induced neuronal activation, leading to a more pronounced upregulation of Arc in SERT-deficient rats in comparison to wild-types (Molteni et al., 2009b). So, these opposite responses in the neuronal activation of PFC induced by acute stress in $SERT^{-/-}$ and $TPH2^{-/-}$ rats further support an important role of the neurotransmitter in stress response. Accordingly, it was recently shown that the 5-HT_2 receptor antagonist ketanserin also blocks *Arc* induction in the PFC in response to a stress paradigm (Benekareddy et al., 2011).

Finally, we found that, despite the increased levels of Bdnf observed in $TPH2^{-/-}$, rats, this compensatory mechanism was not enough to restore the normal ability to react to the acute stress challenge, probably because of the complexity of the system and circuit involved that may be BDNF-independent. Indeed, even if *Bdnf* transcripts were upregulated in both genotypes in response to stress, the effects found in $TPH2^{+/+}$ were stronger than those induced in the $TPH2^{-/-}$ rats. The fact that BDNF is upregulated by stress in the PFC even in the absence of serotonin, while the immediate early genes are not, suggests distinct stress-modulated neural circuits whereby one is serotonergic, and another is serotonin-independent.

Other than immediate early genes and *Bdnf* transcription, another system crucial for stress response is the HPA axis. Interestingly, we observed that corticosterone release was differently modulated by stress in wild-type males and females, and it was affected by lack of serotonin in a sex specific manner. Indeed, as expected from the literature (Adzic et al., 2009; Molteni et al., 2009a) stress exposure induced a strong release of corticosterone immediately after the restraint stress in $TPH2^{+/+}$ male rats and a significant decrease 1 h later, while in females this pattern was not so pronounced with an upregulation specifically in $TPH2^{+/+}$ female rats right after the stress. Since corticosterone level is highly influenced by the estrus cycle (Atkinson and Waddell, 1997) and considering that the females used in our study were not synchronized, the high variability we found in the corticosterone levels might originate from different estrus cycle stages. However, the downstream pathway activated by corticosterone release is very complex: once released from the adrenal glands, it targets the brain where it acts on its receptors widely localized in the cells. While membrane-bound GRs induce a rapid effect by modulating the release of neurotransmitters and mitochondrial activity through the so-called non-genomic pathway, cytosolic receptors are internalized into the nucleus, where, after the binding to specific DNA sequences named glucocorticoid responsive elements, they control the transcription of target genes (Schoneveld et al., 2004; Panettieri et al., 2019). Hence, even if the release of corticosterone following an acute challenge was independent of the serotonin levels in the brain, we analyzed the activation of the genomic pathway focusing on stress0' time point when we found the peak of the release of the hormone in the bloodstream. Moreover, since the females were not synchronized and the estrogens can have a great impact on the activation of the axis, we focused specifically on male rats to better dissect the interplay between serotonin and the genomic pathway of the glucocorticoid receptors. Interestingly, we demonstrated that the internalization into the nucleus of the GRs induced by the exposure to an acute stressor happened specifically in $TPH2^{+/+}$, but not in $TPH2^{-/-}$ rats. Therefore, the absence of serotonin in the brain seems not to influence the release of the hormone from the adrenal glands, but it interferes with the activation of the genomic pathway at the central level. In line, it has been demonstrated that the modulation of the serotonergic system by chronic treatment with antidepressant drugs enhances the translocation of GRs to the nuclear compartment (Molteni et al., 2009a). Since the direct influence of central serotonin on corticosterone release after acute stress is excluded, another mechanism linking serotonin and GR functionality should exist. Most likely

the cross-talk between these two systems appears at the level of interaction between downstream pathways of serotonin receptors with GR phosphorylation. Indeed, it has been demonstrated that the entry of GR to the nucleus is facilitated by the phosphorylation of specific serines (Wang et al., 2002) via activation of MAPKs and CDKs pathways (Galliher-Beckley and Cidlowski, 2009). Since the activity of the MAPKs is modulated by 5-HT_{2A} and $5HT_{1A}$ receptors (Banes et al., 1999; Errico et al., 2001), the blunted activation of serotonin receptor signaling in the absence of ligand may be responsible for the lack of GR phosphorylation and its subsequent translocation to the nucleus upon acute stress. Furthermore, it has been shown that GR phosphorylation is also controlled by BDNF signaling (Arango-Lievano et al., 2015) and we found that $TPH2^{-/-}$ rats showed a reduced *Bdnf* expression after the exposure to 1 h of acute restraint stress compared to the wild-type counterpart. This may lead to a decreased ability of the neurotrophin-related pathways to phosphorylate GRs. Furthermore, the DNA sequences to which GRs binds are characterized by the presence of GC enriched binding sites that are targeted by transcription factors such as MAZ1 and SP1. These transcription factors in the proximity to GREs can potentiate the GR binding and therefore, enhance the transcriptional activation of target genes (Datson et al., 2011). Here, in line with the increase of GR in the nucleus, we observed increased levels of MAZ1 in $TPH2^{+/+}$ stressed animals contrasting with a slight decrease in $TPH2^{-/-}$ stressed animals. This data suggests that the reduced activation of the genomic pathway in $TPH2^{-/-}$ rats in response to the acute challenge may be caused not only by the lack of GRs translocation per se but also by the reduced levels of this cofactor. On the contrary, SP1 was not altered after stress exposure. Hence, these two cofactors may influence the interaction between GRs and their responsive elements with different timing. Furthermore, even if both MAZ1 and SP1 can interact with GRs, SP1 seems to be mainly related to mineralocorticoid receptors (Meinel et al., 2013) suggesting its potential implication in physiological conditions and not in response to an acute challenge.

As mentioned, the GR, as a nuclear receptor, acts as a transcriptional regulator for genes carrying the GRE in their regulatory sequence (Gray et al., 2017). In line, we recently demonstrated that the translocation into the nucleus is paralleled by an up-regulation of the expression of genes containing the GRE such as *Gadd45β*, *Sgk1*, *Nr4a1*, and *Dusp1* (Calabrese et al., 2020). Here, we observed that GR responsive genes were activated more in $TPH2^{+/+}$ than in $TPH2^{-/-}$ rats. In particular, *Dusp1* mRNA was increased specifically in $TPH2^{+/+}$ while no

changes were observed in TPH2$^{-/-}$ rats. Similarly, *Gadd45β* and *Sgk1* mRNAs were increased in both genotypes, but more in TPH2$^{+/+}$ than in TPH2$^{-/-}$ rats. This suggests the ability of serotonin to interfere with their transcription. Indeed, several studies showed that pharmacological or genetic alterations of the serotonergic system can modulate their expression (Gonzalez-Nicolini and McGinty, 2002; Duric et al., 2010). However, other glucocorticoid responsive genes were equally upregulated after the acute challenge in the two genotypes. This could be due to other factors that can influence their expression and that may contribute to the stress response (Lang et al., 2014; Zannas et al., 2016). Furthermore, it could not be excluded that the absence of serotonin may modify the epigenetic make-up of some GR related genes via the histone-serotonylation mechanism (Farrelly et al., 2019), and therefore their transcription after an acute challenge. Finally, serotonin is involved in the control of various physiological functions, and, among them, the circadian system is braided with the neurotransmitter both anatomically and genetically (Deurveilher and Semba, 2005). Furthermore, it has been demonstrated that genes involved in the clock gene machinery can be affected by acute stress exposure (Yamamoto et al., 2005). Interestingly, *Per1* expression showed a similar pattern as *Gadd45β* and *Nr4a1*, with increased transcription in TPH2$^{+/+}$ compared to TPH2$^{-/-}$ rats exposed to stress. On the contrary, *Per2* mRNA levels were increased more in TPH2$^{-/-}$ than in TPH2$^{+/+}$ rats. This different pattern could be due to a different responsiveness of *Per1* and *Per2* to GRs. Indeed, while *Per1* carries a canonical GRE, *Per2* has an intronic binding sequence that confers GR responsiveness to the gene (So et al., 2009). Moreover, *Rev-erbα* expression was up-regulated by stress in TPH2$^{+/+}$ but not in TPH2$^{-/-}$ rats while *Rev-erbβ* expression showed a basal increase TPH2$^{-/-}$ rats while a normalization occurred after the acute stress. Since *Rev-erbs* are responsible for the proper interlocking feedback loop in the clock machinery (Lowrey and Takahashi, 2011), the modulations observed could indicate alterations in the circadian rhythm at basal level and in the feedback to clock gene activation after the stress.

All in all, our data suggest that serotonin deficiency in the brain modulates the neuroplastic mechanisms. Indeed, at basal level, the increase in Bdnf levels from early adolescence until adulthood is probably a compensatory mechanism for the trophic support physiologically provided by the serotonin. However, in response to a stressful condition, the system is not able to properly respond by setting in motion the strategies to cope with an acute challenge. Indeed, the activation of neuroplastic mechanisms that occurred in wild-type rats, were only partially activated in a condition of absence of serotonin in the brain. Accordingly, also the

activation of the genomic pathway of the glucocorticoid was dumped by the lack of the neurotransmitter at central level. Finally, serotonin depletion affected clock gene activity at basal level and in response to the acute challenge, suggesting a potential link between serotonin, stress and circadian rhythms.

4.3. TPH1 $^{-/-}$ rats

4.3.1. Introduction

As for the TPH2, localized in the brain, also the isoform 1 of the rate limiting enzyme for the production of serotonin in the peripheral compartments have been linked to the manifestation of different psychopathologies (Viikki et al., 2010; Goenjian et al., 2012; Wigner et al., 2018). Accordingly, low levels of the monoamine have been found in the peripheral blood of people suffering from depression (Quintana, 1992; Maurer-Spurej et al., 2004). However, the mechanisms through which peripheral serotonin could influence the brain functionality are not clear yet also considering the inability of the serotonin to pass through the blood-brain barrier (Berger et al., 2009) during adulthood. Since 2003, when Walther and colleagues discovered the presence of two different isoforms of the TPH enzyme, preclinical studies have been conducted on TPH2 or TPH1 knockout murine models (Walther et al., 2003). In this context, while TPH2 allows a better understanding of serotonin roles in the brain, TPH1 knockout rodents are useful tools not only to understand the functionality of the monoamine in peripheral organs, but they could also help to evaluate its influence on brain functionality. TPH1 knockout mice, as expected, show a complete deficiency of serotonin in the peripheral compartments but normal levels of neuronal serotonin (Cote et al., 2003; Walther and Bader, 2003). Moreover, this animal model shows alterations in cardiac functionality with respiratory distress and blood circulation irregularities at adulthood but no other anatomical abnormalities in peripheral organs (Cote et al., 2003). Furthermore, adult TPH1$^{-/-}$ mice showed impairments in insulin secretion from pancreatic β-cells (Paulmann et al., 2009) but also in energy metabolism regulation (Sumara et al., 2012; Crane et al., 2015; Choi et al., 2018). Finally, even if it has been demonstrated that the lack of peripheral serotonin can influence brain functionality specifically modulating locomotor activity (Suidan et al., 2013), other behaviors, more likely linked to psychopathologies, have not been investigated yet.

On these bases and to better dissect the impact of low levels of peripheral serotonin on brain functionality, we employed TPH1$^{-/-}$ rats generated at the Gene Editing Rat Resource Center of the Medical College of Wisconsin. As first step, we measured serotonin and tryptophan levels in different peripheral organs to confirm the lack of TPH1 functionality. Moreover, we exposed TPH1$^{+/+}$ and TPH1$^{-/-}$ rats to the elevated plus maze test to evaluate their anxious-like behavior,

a common feature of a wide range of different psychopathologies linked to TPH1 polymorphisms (Karpov et al., 2016).

Finally, considering the strong role of the environment in revealing the molecular alterations and in the onset of psychopathologies we evaluated the impact of peripheral hyposerotoninergia in stress responsiveness. In particular, we employed the same experimental paradigm adopted for $TPH2^{-/-}$ rats and we exposed these animals to one single session of acute restraint stress. As for the previous experiments, we evaluated the impact of low serotonin levels and of the acute stress on *Bdnf* expression, as well as on other neuroplasticity genes. Moreover, to study the activation of the glucocorticoid genomic pathway that we found to be altered in TPH2 knockout rats, we measured the expression of different glucocorticoid responsive genes.

As for $SERT^{-/-}$ and $TPH2^{-/-}$ analyses, all the gene expression analyses were conducted in the PFC, a brain region highly responsive to serotonin levels and to the environment (Duman and Monteggia, 2006; Brivio et al., 2020b).

4.3.2. Results

4.3.2.1. Serotonin levels are dramatically reduced in peripheral organs of TPH1$^{-/-}$ rats

We first analyzed the levels of both serotonin and of its precursor tryptophan in different peripheral organs. As expected, serotonin levels were dramatically reduced in several tissues of TPH1$^{-/-}$ rats when compared to TPH1$^{+/+}$ (blood: -683 ng/mL $p<0.01$ vs TPH1$^{+/+}$; pineal gland: -65398pg $p<0.01$ vs TPH1$^{+/+}$; spleen: -1868pg/mg $p<0.001$ vs TPH1$^{+/+}$; duodenum: -2356pg/mg $p<0.01$ vs TPH1$^{+/+}$; ileum: -1396pg/mg $p<0.01$ vs TPH1$^{+/+}$; colon: 1403pg/mg $p<0.01$ vs TPH1$^{+/+}$; liver: -241pg/mg $p<0.01$ vs TPH1$^{+/+}$; Student's t test) (Table 5).

Serotonin levels

Organ	**TPH1$^{+/+}$**	**TPH1$^{-/-}$**
Blood (ng/mL)	725±133	42±8 **
Pineal gland (pg)	66013±7232	615±89 **
Spleen (pg/mg)	2055±47	187±45 ***
Duodenum (pg/mg)	2372±400	16±3 **
Ileum (pg/mg)	1412±208	16±2 **
Colon (pg/mg)	1418±211	15±1 **
Liver (pg/mg)	261±29	20±2 **

Table 5: serotonin levels in peripheral organs of TPH1$^{+/+}$ and TPH1$^{-/-}$ rats. The data are presented as mean±standard error of the mean (SEM) of at least 5 independent determinations **p<0.01; ***p<0.001 vs TPH1$^{+/+}$; Student's t test.

4.3.2.2. Tryptophan levels are modulated specifically in the pineal gland and in the liver of TPH1$^{-/-}$ rats

While serotonin concentration was homogenously reduced in peripheral organs, we found that the levels of its precursor tryptophan were increased specifically in the pineal glands (+9201pg $p<0.01$ vs TPH1$^{+/+}$; Student's t test) and reduced in the liver (-1329pg/mg $p<0.05$ vs TPH1$^{+/+}$; Student's t test) (Table 6). Conversely, we did not find any significant changes in the blood, spleen, and in the different gut compartments (Table 6).

Tryptophan levels

Organ	**TPH1$^{+/+}$**	**TPH1$^{-/-}$**
Blood (ng/mL)	6815±736	6751±727
Pineal gland (pg)	5023±1675	14224±812 **
Spleen (pg/mg)	8589±332	10579±1766
Duodenum (pg/mg)	5987±576	5702±512
Ileum (pg/mg)	8401±1356	7076±813
Colon (pg/mg)	3356±523	3851±220
Liver (pg/mg)	5061±343	3732±442 *

Table 6: tryptophan levels in peripheral organs of TPH1$^{+/+}$ and TPH1$^{-/-}$ rats. The data are presented as mean±standard error of the mean (SEM) of at least 4 independent determinations *$p<0.05$; **$p<0.01$ vs TPH1$^{+/+}$; Student's t test.

4.3.2.3. TPH1$^{-/-}$ rats showed a reduced anxiety in the elevated plus maze test

Anxiety is a common feature of different psychopathologies which have been linked to TPH1 polymorphisms (Karpov et al., 2016). Hence, here we analyzed the anxiety levels of TPH1$^{+/+}$ and TPH1$^{-/-}$ rats by exposing the animals to the elevated plus maze test.

Interestingly, the lack of peripheral serotonin induced a reduction in the anxiety behavior. Indeed, TPH1$^{-/-}$ rats spent more time in the open arms (+17.36s vs TPH1$^{+/+}$ $p<0.01$; Student's t test; Figure 27A) and in the center of the maze (+22.24 $p<0.05$ vs TPH1$^{+/+}$ $p<0.01$; Student's t test; Figure 27C) while less time in the closed arms (-40.38s $p<0.01$ vs TPH1$^{+/+}$ $p<0.01$; Student's t test; Figure 27B). Moreover, as shown in Figure 27D, they showed a reduced latency to the first entry in the open arms (-10.03s $p<0.05$ vs TPH1$^{+/+}$ $p<0.01$; Student's t test).

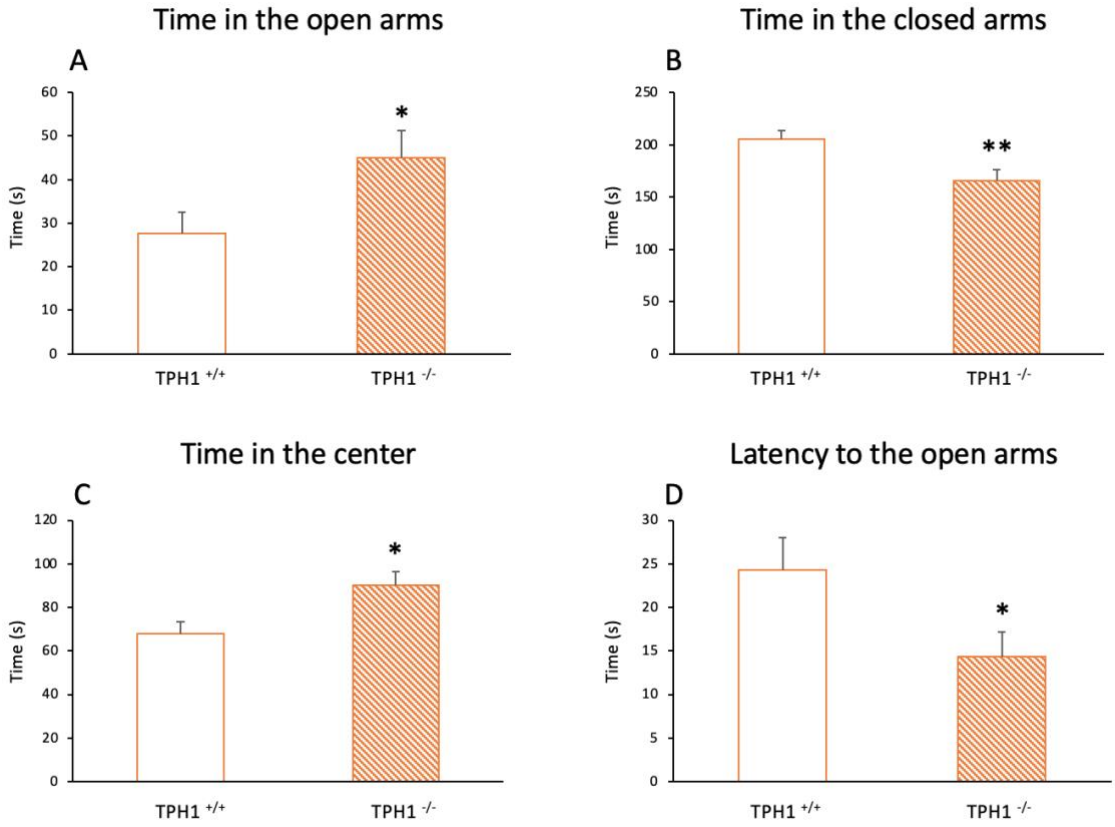

Figure 27: elevated plus maze test results. Time spent in the open arms (A), in the closed arms (B) and in the center (C) and latency to the first entrance in the open arms (D). Data are presented as mean±standard error of the mean (SEM) of at least 21 independent determinations. *$p<0.05$; **$p<0.01$ vs TPH1$^{+/+}$; Student's t test.

4.3.2.4. TPH1$^{-/-}$ rats showed an increased neuroplasticity at basal level but an impaired *Bdnf* transcription after acute stress

Seen the strict interplay between the serotonergic system and the neurotrophic mechanisms and considering the altered *Bdnf* expression we observed, both at basal level and after environmental stimulation, in SERT$^{-/-}$ and TPH2$^{-/-}$ rats, here, we measured the expression levels of total *Bdnf*, of *Bdnf* long 3'UTR and of the two most diffuse isoforms in the brain, the isoforms IV and VI, both at basal level and after one single session of acute restraint stress in TPH1 $^{-/-}$ rats.

Interestingly, we found a significant genotype X stress interaction on total *Bdnf* mRNA levels ($F_{(1-21)}$ = 4.806 p<0.05; two-way ANOVA). In line, we found an upregulation in the total form of the neurotrophin transcription at basal level in TPH1$^{-/-}$ rats (+56% p<0.05 vs TPH1$^{+/+}$/naïve; Fisher's PLSD) compared to TPH1$^{+/+}$ animals. Moreover, we found that its transcription was positively modulated after the acute challenge specifically in TPH1$^{+/+}$ (+52% p<0.05 vs TPH1$^{+/+}$/naïve; Fisher's PLSD) while not in TPH1$^{-/-}$ rats (Figure 28A).

Furthermore, as shown in figure 28B, C we found a similar transcription pattern for *Bdnf* long 3'UTR and *Bdnf* isoform IV (*Bdnf* long 3'UTR: genotype X stress interaction: $F_{(1-19)}$ = 5.817 p<0.05; *Bdnf* isoform IV: $F_{(1-19)}$ = 5.345 p<0.05; two-way ANOVA). Indeed, the lack of peripheral serotonin induced an increase in their mRNA levels in TPH1$^{-/-}$ animals (*Bdnf* long 3'UTR: +35% p<0.05; *Bdnf* isoform IV: +32% p<0.01 vs TPH1$^{+/+}$/naïve; Fisher's PLSD) and, one hour of restraint stress upregulated the pool of the long transcripts and the isoform IV expression in wild-type rats (*Bdnf* long 3'UTR: +33% p<0.05; *Bdnf* isoform IV: +43% p<0.05 vs TPH1$^{+/+}$/naïve; Fisher's PLSD) while we did not find any further increase for TPH1$^{-/-}$ stressed rats.

Finally, we observed a genotype X stress interaction on *Bdnf* isoform VI transcription ($F_{(1-20)}$ = 5.165 p<0.05; two-way ANOVA) with an upregulation of the isoform VI expression in TPH1$^{-/-}$ naïve animals (+42% p<0.05 vs TPH1$^{+/+}$/naïve; Fisher's PLSD) which was normalized after the acute challenge (-27% p<0.05 vs TPH1$^{+/+}$/naïve; Fisher's PLSD) (Figure 28D).

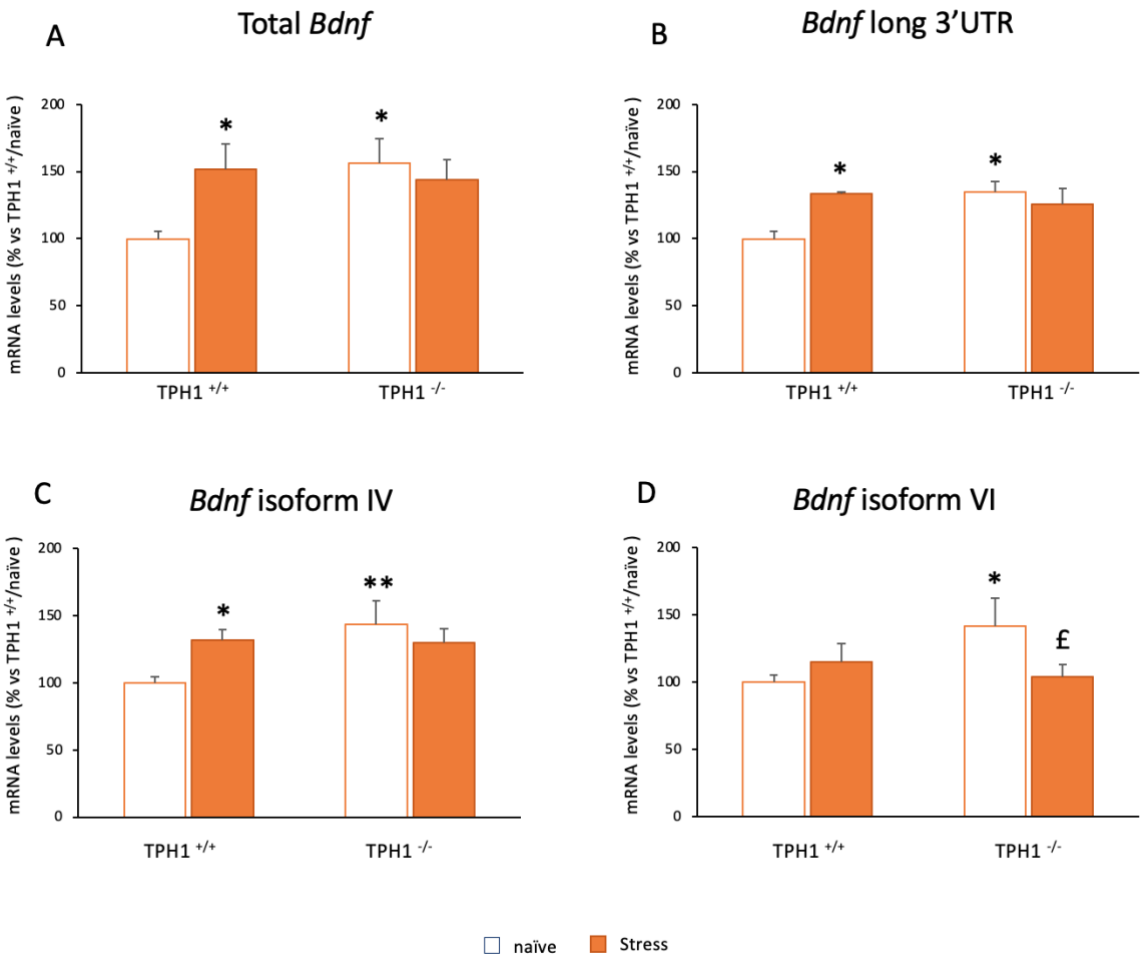

Figure 28: analyses of total *Bdnf* (A), *Bdnf* long 3'UTR (B), *Bdnf* isoforms IV (C) and VI (D) mRNA levels in the PFC of TPH1$^{+/+}$ and TPH1$^{-/-}$ male adult rats exposed to one single session of acute restraint stress and sacrificed right after the stress. The data are presented ad percent change of TPH1$^{+/+}$/naïve and are expressed as mean±standard error of the mean (SEM) of at least 4 independent determinations. *p<0.05; **p<0.01 vs TPH1$^{+/+}$/naïve; £p<0.05 vs TPH1$^{-/-}$/naïve two-way ANOVA with Fisher's PLSD.

4.3.2.5. The enhancement in the immediate early gene *Arc* expression due to the acute stress exposure is blunted in TPH1$^{-/-}$ rats

Seen the specific upregulation of the neurotrophin *Bdnf* after the acute challenge in TPH1$^{-/-}$ rats, we also evaluated the expression of the two immediate early genes *Arc* and *Cfos* which are normally upregulated after acute stimuli.

Interestingly, we found an effect of the stress ($F_{(1-22)}$ = 4.963 p<0.05; two-way ANOVA) and a genotype X stress interaction ($F_{(1-22)}$ = 4.518 p<0.05; two-way ANOVA) on *Arc* expression. Indeed, as shown in Figure 29A, we found an upregulation of its mRNA levels specifically in TPH1$^{+/+}$ stressed rats (+57% p<0.01 vs TPH1$^{+/+}$/naïve; Fisher's PLSD) while no changes in the knockout counterpart. Consequently, *Arc* expression was significantly higher in TPH1$^{+/+}$ stressed rats when compared to TPH1$^{-/-}$ rats exposed to 1h of restraint (+43% p<0.05 vs TPH1$^{-/-}$/stress Fisher's PLSD).

On the contrary, *Cfos* expression was affected nor by the stress neither by the genotype (Figure 29B).

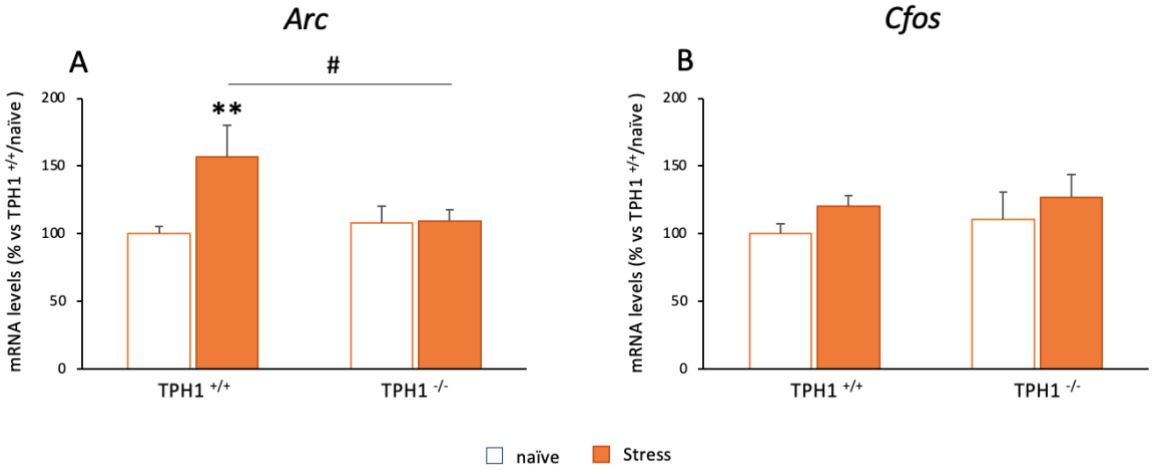

Figure 29: analyses of *Arc* (A) and *Cfos* (B) mRNA levels in the PFC of TPH1$^{+/+}$ and TPH1$^{-/-}$ male adult rats exposed to one single session of acute restraint stress and sacrificed right after the stress. The data are presented ad percent change of TPH1$^{+/+}$/naïve and are expressed as mean±standard error of the mean (SEM) of at least 5 independent determinations. **p<0.01 vs TPH1$^{+/+}$/naïve; #p<0.05 vs TPH1$^{+/+}$/stress two-way ANOVA with Fisher's PLSD.

4.3.2.6. Glucocorticoid responsive genes upregulation is blunted in TPH1$^{-/-}$ rats exposed to stress

Glucocorticoids play a crucial role in stress response, and we found that the activation of the genomic pathway of the glucocorticoids after stress exposure was impaired in rats lacking serotonin in the brain. Therefore, to evaluate the involvement of peripheral serotonin in this mechanism, here we measured the expression of the glucocorticoid responsive genes in TPH1$^{-/-}$ rats.

Interestingly, as shown in Figure 30A, we found an effect of the stress on *Dusp1* expression ($F_{(1-21)}$ = 7.478 p<0.05; two-way ANOVA) with an upregulation induced by stress that happened specifically in TPH1$^{+/+}$ stressed rats (+27% p<0.05 vs TPH1$^{+/+}$/naïve: Fisher's PLSD) while not in TPH1$^{-/-}$ rats exposed to the acute challenge.

Similarly, we found an effect of the genotype and of the stress on *Fkbp5* mRNA levels (genotype: $F_{(1-22)}$ = 6.241 p<0.05; stress: $F_{(1-22)}$ = 5.874 p<0.05 two-way ANOVA) and a significant genotype X stress interaction on *Nr4a1* expression ($F_{(1-23)}$ = 6.569 p<0.05; two-way ANOVA). Indeed, we found a specific upregulation of both *Fkbp5* and *Nr4a1* after the stress in TPH1$^{+/+}$ rats (*Fkbp5*: +35% p<0.05; *Nr4a1*: +30% p<0.05 vs TPH1$^{+/+}$/naïve; Fisher's PLSD) while no changes in TPH1$^{-/-}$ rats (Figures 30B, C).

Moreover, *Sgk1* expression was modulated by the stress ($F_{1-23)}$ = 76.873 p<0.001 two-way ANOVA) with an upregulation of its expression both in TPH1$^{+/+}$ (+95% p<0.001 vs TPH1$^{+/+}$/naïve; Fisher's PLSD) and TPH1$^{-/-}$ rats (+71% p<0.001 vs TPH1$^{-/-}$/naïve; Fisher's PLSD). However, this increase was more robust in TPH1$^{+/+}$ than in TPH1$^{-/-}$ stressed rats (+18% p<0.05 vs TPH1$^{+/+}$/stress; Fisher's PLSD) (Figure 30D).

Finally, as shown in Figures 30E, F we observed and effect of the stress on *Gadd45β* and *Gilz* expression (*Gadd45β*: $F_{(1-23)}$ = 33.405 p<0.001; *Gilz*: $F_{(1-23)}$ = 86.627 p<0.001; two-way ANOVA) with an equal upregulation of their expression in TPH1$^{+/+}$ (*Gadd45β*: +60% p<0.001; *Gilz*: +103% p<0.001 vs TPH1$^{+/+}$/naïve; Fisher's PLSD) and in TPH1$^{+/+}$ rats (*Gadd45β*: +65% p<0.001; *Gilz*: +71% p<0.001 vs TPH1$^{-/-}$/naïve; Fisher's PLSD).

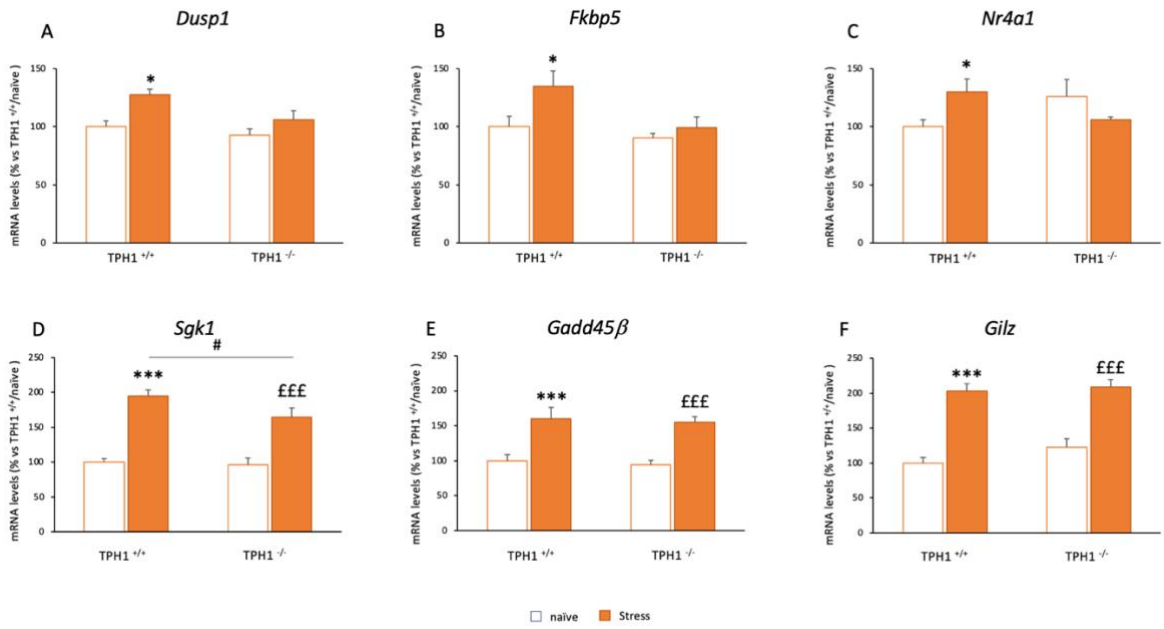

Figure 30: analyses of *Dusp1* (A), *Fkbp5* (B), *Nr4a1* (C), *Sgk1* (D), *Gadd45β* (E) and *Gllz* (F) mRNA levels in the PFC of TPH1$^{+/+}$ and TPH1$^{-/-}$ male adult rats exposed to one single session of acute restraint stress and sacrificed right after the stress. The data are presented ad percent change of TPH1$^{+/+}$/naïve and are expressed as mean±standard error of the mean (SEM) of at least 5 independent determinations. *p<0.05; ***p<0.001 vs TPH1$^{+/+}$/naïve; £££p<0.001 vs TPH1$^{-/-}$ /naïve #p<0.05 vs TPH1$^{+/+}$/stress two-way ANOVA with Fisher's PLSD.

4.3.3. Discussion

The results obtained from the present study conducted on TPH1$^{-/-}$ rats highlight the ability of peripheral serotonin of altering brain functions resulting in changes in the behavior as well as in molecular systems both in basal conditions and after the exposure to an acute restraint stress.

In peripheral organs, as expected, we found that the deletion of the TPH1 gene resulted in a dramatic reduction of serotonin levels in blood, pineal glands, spleen, duodenum, ileum, colon and in the liver. Accordingly, similar results have been found in TPH1$^{-/-}$ mice (Cote et al., 2003; Walther et al., 2003). Moreover, tryptophan levels were not altered in blood, spleen and in the gut while we found an increase of this serotonin precursor in the pineal gland. Interestingly, it has been demonstrated that the pineal gland, which is outside the blood-brain barrier, is enriched in both high- and low-affinity tryptophan transporter (Gutiérrez et al., 2003) and this may increase the uptake of the unexploited amount of tryptophan from the periphery shifting its metabolism from serotonin production to the kynurenine pathway, which represents about 95% of dietary tryptophan metabolism in the body (Botting, 1995). Furthermore, we found a downregulation of the amino acid tryptophan levels in the liver. Since the first step of tryptophan metabolism to kynurenines in the periphery occurs through the action of the tryptophan 2,3-dioxygenase (TDO), an enzyme almost exclusively located in the hepatic cells that can control free tryptophan concentration in the blood (Badawy, 1977), here, we could speculate that the unused tryptophan could be rapidly transformed in the liver thank to the action of the TDO enzyme.

After having confirmed that the deletion of the isoform 1 of the TPH enzyme resulted in a dramatic reduction of serotonin levels in peripheral organs, and to better understand the impact of this alteration on brain functions in the field of psychopathologies, we have evaluated the anxiety-like behavior of these animals by exposing them to the elevated plus maze test. Results from this behavioral test indicated that TPH1$^{-/-}$ rats are less anxious when compared to TPH1$^{+/+}$. Indeed, they spent more time in the open arms and in the center of the apparatus while less time in the closed arms which are considered a safer part of the plus maze. Moreover, they showed a reduced latency to the first entry in the open arms. Although it has been shown that the lack of peripheral serotonin is able to alter locomotor activity (Suidan et al., 2013), no study to date had evaluated whether the altered function of TPH1 could alter other behaviors more related to psychiatric pathologies. Despite the evidence

relating the altered functionality of the TPH1 with different mood disorders characterized by an increase in anxiety levels (Viikki et al., 2010; Goenjian et al., 2012; Wigner et al., 2018), here we found that the lack of peripheral serotonin in TPH1$^{-/-}$ rats seems to have a positive impact on this behavior at basal level.

However, in the context of psychiatric illnesses, often the genetic background acts as a risk factor and different molecular alterations could be unmasked only after an environmental stimulus. Accordingly, as shown in the previous chapter, the lack of central serotonin induced impairments in the activation of different systems only after an acute stress. Hence, here we exposed TPH1$^{+/+}$ and TPH1$^{-/-}$ rats to one single session of acute restraint stress and we evaluated if also low peripheral serotonin levels could interfere with the activation of those systems involved in stress response and altered in TPH2$^{-/-}$ stressed rats.

In particular, we firstly focus on the neurotrophic mechanisms, and we found that the total form of *Bdnf* was upregulated at basal level in TPH1$^{-/-}$ rats and this increase was sustained by the transcription of the pool of the long transcripts of the neurotrophin and by *Bdnf* isoform IV and VI. However, while TPH1$^{+/+}$ stressed rats showed an activation of the system with an increase in total *Bdnf* mRNA levels as well as of *Bdnf* long 3'UTR and of *Bdnf* isoform IV, we did not find any further increase in rats lacking peripheral serotonin. Remarkably, we observed similar results in rats lacking central serotonin where the stress induced a stronger increase in *Bdnf* expression in wild-type rats when compared to TPH2$^{-/-}$ rats. Thus, we could hypothesize that not only central, but also peripheral serotonin is able to interfere with the activation of *Bdnf* machinery after stress exposure. In line with these results, by measuring the expression of the immediate early genes *Arc* and *Cfos* which are implicated in acute stress response (Ons et al., 2004; Molteni et al., 2009b), we found an upregulation of *Arc* specifically in TPH1$^{+/+}$ rats exposed to stress while the lack of serotonin in peripheral compartments shut down its activation. On the contrary, we did not find changes in *Cfos* expression nor in TPH1$^{+/+}$ neither in TPH2$^{-/-}$ rats. Hence these results suggest an impairment in the reactivity of the brain to acute stimuli.

To further support this evidence, we evaluated the expression pattern of the glucocorticoid responsive genes that are normally transcribed after acute stress exposure and the release of corticosterone (Schoneveld et al., 2004; Panettieri et al., 2019) and that we found to be altered in TPH2$^{-/-}$ animals. Similar to what happens in rats with no brain serotonin, here we found that *Dusp1*, *Fkbp5* and *Nr4a1* were upregulated specifically in wild-type rats exposed to

stress while their transcription was completely blunted in knockout animals. Moreover, *Sgk1* was upregulated in both genotypes, but this increase was higher in TPH1$^{+/+}$ rats. Finally, *Gadd45β* and *Gilz* were equally upregulated after stress both in TPH1$^{+/+}$ and TPH1$^{-/-}$ rats. Despite other analyses should confirm this hypothesis, here we can speculate that the absence of peripheral serotonin could interfere with the corticosterone release consequently inhibiting the whole downstream pathway. In line, the adrenal gland, from which corticosterone is released, presents serotonin receptors and their agonism stimulates the release of this hormone (Hegde and Eglen, 1996). Moreover, serotonin can stimulate the release of the corticotropin-releasing hormone (CRH) from the hypothalamic neurons and of the Adreno Cortico Tropic Hormone (ACTH) from the pituitary gland, both central regions lacking the blood-brain barrier and exposed to peripheral serotonin (Jørgensen et al., 2002; Chen and Miller, 2012). As for TPH2$^{-/-}$ rats, not all these genes have the same activation, and this could be due to other stress-related factors that could activate their transcription (Lang et al., 2014; Zannas et al., 2016).

All in all, these data suggest that, despite the presence of two distinct pools of serotonin in the brain and at the periphery at adulthood, alterations in peripheral serotonergic levels can affect brain functionality. This cross talk between peripheral serotonin and the CNS could take origin during the development. Indeed, the blood-brain barrier represents a clear boundary at adulthood, but during the first stages of life it is immature and different molecules, included serotonin, can pass through it (Ribatti et al., 2006). Therefore, the reduced amount of serotonin reaching the brain during the first period of life could interfere with the correct formation of neuronal circuits and therefore inhibit their correct functioning. Another hypothesis that could justify the influence of peripheral serotonin on the brain functionality is the involvement of the gut-brain axis, which is greatly sensitive to the serotonin levels: low levels of serotonin could indeed interfere with the proper cross talk via the vagus nerve as well as by altering the microbiome composition (Nanthakumaran et al., 2020).

5. General discussion

During my PhD program, I evaluated the role of the serotoninergic system in modulating brain functionality. Specifically, we assessed how alterations of this system could contribute to the increased susceptibility to develop psychiatric disorders. To this aim, we conducted analyses both at behavioral as well as at molecular level in different animal models with specific alterations of the serotoninergic systems and we characterize them both at basal level and in more dynamic stations by altering their environment.

In particular, we took advantage of $SERT^{-/-}$ rats, a well-established animal model of depression characterized by a pathological-like phenotype. Indeed, the absence of the serotonin transporter in the whole-body results in a pathological phenotype in terms of anhedonia assessed with the sucrose consumption test and of anxiety-like behavior measured in the elevated plus maze test. Moreover, this animal model is characterized by different molecular pathways alterations in the brain such as a reduced expression of neuroplasticity markers, but also changes in spine density markers and in the GABAergic and glutamatergic systems (Olivier et al., 2008; Calabrese et al., 2010b, 2013; Molteni et al., 2010; Guidotti et al., 2012; Brivio et al., 2019). This is in line with human evidence highlighting the link between genetic alterations of the SERT gene with the increased susceptibility to develop mood disorders (Caspi et al., 2003; Munafò et al., 2009; Risch et al., 2009; Karg et al., 2011; Sharpley et al., 2014; Bleys et al., 2018; Culverhouse et al., 2018). More recently, however, it has been described how, in humans, the polymorphism of this gene can induce a greater sensitivity to the environment rather than an increased susceptibility specifically to adverse stimuli. This could result on one side to better outcomes in positive contexts and, on the other hand, to worst consequences in negative circumstances (Belsky et al., 2009; Pluess, 2017). Here, to explore this hypothesis in a preclinical model and to understand the possible molecular bases responsible for this amplified reaction to external stimuli, we exposed $SERT^{+/+}$ and $SERT^{-/-}$ rats to one month of normal or enriched environment.

In line with previous evidence, we confirmed the depressive-like behavior derived from the lack of SERT functionality at basal level. Moreover, we supported previous literature results demonstrating the involvement of the neuroplastic mechanisms, of the GABAergic system and of spine functionality in shaping this pathological behavior (Guidotti et al., 2012). Interestingly, we found that the enriched environment was able to normalize these alterations in $SERT^{-/-}$ rats by restoring a normal behavior. Furthermore, in line with the vantage sensitivity theory, we found that the positive rearing conditions were specifically effective in animals lacking the

SERT, while it did not affect wild-type rats. This further supports the greater influence of the external contexts in organisms with SERT functionality alterations.

Besides the SERT allelic variants, also other polymorphisms on genes coding for other players involved in the serotoninergic system have been associated with a major probability to develop psychiatric symptoms (Ottenhof et al., 2018; Viikki et al., 2010; Wigner et al., 2018). In particular, the TPH2 gene has different SNPs that could alter its expression and be responsible for this susceptibility. Hence, another animal model considered in this thesis is the $TPH2^{-/-}$ rats. The lack of the TPH isoform located in the brain results in a complete absence of central serotonin, in a growth retardation during the early stages of life until the weaning while no other developmental abnormalities are observed (Kaplan et al., 2016). Moreover, adult $TPH2^{-/-}$ rodents show a clear aggressive-like behavior and a reduced anxiety (Mosienko et al., 2012; Peeters et al., 2019). Therefore, to better evaluate the role of brain-derived serotonin in the organism development and to dissect how its alterations could predispose to psychiatric disorders, we perform a basal characterization of this animal model from birth until adulthood focusing on neuroplastic mechanisms which are closely linked to the serotonergic system (Homberg et al., 2014). Interestingly, we found that at post-natal day 10, the lack of central serotonin induced a downregulation of *Bdnf* expression. However, after the complete closure of the blood-brain barrier, which in rodents happens around post-natal day 12 (Ribatti et al., 2006), and the formation of two distinct pools of serotonin, we found increased levels of the neurotrophin that persisted until adulthood. Hence, we could hypothesize that in basal conditions the serotonin is supplied to the brain by peripheral sources until the complete closure of the blood-brain barrier when its absence is somehow compensated by the upregulation of the major neurotrophin in the brain, the brain-derived neurotrophic factor. Even if this compensation at the basal level could be considered as positive, the exposure to negative environmental stimuli may unmask some defective mechanisms that are not set in motion correctly. So, to evaluate this aspect, we exposed adult $TPH2^{-/-}$ rats to an acute challenge and we found that central serotonin deficient rats were not able to activate different systems normally set in motion after environmental stimuli. In particular, we found that *Bdnf* transcription, which, as expected, was upregulated in $TPH2^{+/+}$ stressed rats, was not further increased in $TPH2^{-/-}$ rats exposed to the restraint stress. Accordingly, also the upregulation in the immediate early genes expression that we observed in $TPH2^{+/+}$ rats exposed to the restraint stress was reduced in knock-out rats.

Finally, we also evaluated the activation of the HPA axis, which is normally set in motion after stressful stimuli and that plays a major role to allow the organism to cope with the stress (McEwen et al., 2015). Similar to the other systems investigated, we found that the activation of the axis induced by the acute challenge was impaired in TPH2$^{-/-}$ rats. Indeed, we found that the internalization of the GR into the nucleus, which is required for the activation of the glucocorticoid genomic pathway, was completely blunted in absence of the neurotransmitter in the brain. As consequence, we observed deficits in the expression of the glucocorticoid responsive genes. Altogether, these results suggest how the absence of central serotonin in TPH2$^{-/-}$ rats is covered by other systems at basal level, but its negative effects are unmasked when the system is called to cope with more dynamic situations such as the exposure to acute stressors.

Considering these results and the clinical evidence highlighting the link between TPH1 isoform polymorphisms and psychopathologies (Viikki et al., 2010; Wigner et al., 2018), we decided to evaluate if also peripheral serotonin could shape brain functionality both at basal level and after an acute stress. Hence, we took advantage of the TPH1$^{-/-}$ rats which showed a dramatic reduction of serotonin levels in peripheral compartments during adulthood. Intriguingly, we found that not only central but also peripheral serotonin can modulate brain functionality. Indeed, we found that TPH1$^{-/-}$ rats showed a reduced anxiety-like behavior during the elevated plus maze test. Moreover, this positive behavioral outcome was supported by increased levels of the neurotrophin *Bdnf*. Nevertheless, as happened in TPH2$^{-/-}$ rats, also TPH1$^{-/-}$ rats showed impairments in the proper response to the acute challenge in terms of *Bdnf* and immediate early genes expression as well as in the transcription of the glucocorticoid responsive genes. Hence, these results suggest how peripheral derived serotonin can affect brain functionality and responses to the environment. This could derive from developmental abnormalities that happened before the maturation of the blood-brain barrier or via the gut-brain axis which is also implicated per se in the manifestation of psychopathologies.

All in all, the results obtained from SERT$^{-/-}$, TPH2$^{-/-}$, and TPH1$^{-/-}$ rats both at basal level and after the exposure to different positive or negative stimuli allow a better understanding of the mechanisms modulated by the serotonin. Moreover, these data better define how an organism with alterations of this system could be more sensitive to the surroundings and be more susceptible to develop psychopathologies. Finally, these data could be the basis to

develop more specific therapeutic approaches involving both pharmacological and psychological tools.

6. Conclusions

In conclusion, during my PhD program I took into consideration three different transgenic animal models with specific alterations of the serotoninergic system and I evaluated how these genetic manipulations could alter their behavior and their brain functionality. In line, we conducted behavioral tests and molecular analyses both in basal conditions as well as after the exposure to positive or negative environmental stimuli.

In particular, we confirmed literature data on the pathological-like behavior of $SERT^{-/-}$ rats and on its molecular alterations in the brain. Interestingly we found that one month of positive environment has been able to normalize these behavioral and molecular alterations.

On the other hand, we found that $TPH2^{-/-}$ and $TPH1^{-/-}$ rats did not show a well-defined pathological behavior at basal levels. However, when they had to cope with a negative stimulus such as one hour of acute restraint stress, we found impairment in the activation of different systems normally set in motion in physiological conditions.

All in all, we proved the usefulness of these transgenic animal models in understanding the implication of serotoninergic alterations in the modulation of brain functionality and in clarifying the potential mechanisms underlined. Furthermore, these results further support the strong involvement of serotonin in brain development and in the control of mood and other behaviors and molecular mechanisms in the brain. Moreover, we give novel insights on the pathways that are affected by the serotoninergic system and that may shape the environment perception and the brain responses to it. Finally, these studies give new perspective on the gene and environment interaction in the field of psychiatric diseases.

7. Bibliography

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