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Research paper

Chronic exposure to imipramine induces a switch from depression-like to mania-like behavior in female serotonin transporter knockout rats: Role of BDNF signaling in the infralimbic cortex

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ABSTRACT

Background: Bipolar disorder (BD) is a highly burdensome psychiatric disorder characterized by alternating states of mania and depression. A major challenge in the clinic is the switch from depression to mania, which is often observed in female BD patients during antidepressant treatment such as imipramine. However, the underlying neural basis is unclear.
Methods: To investigate the potential neuronal pathways, serotonin transporter knockout (SERT KO) rats, an experimental model of female BD patients, were subjected to a battery of behavioral tests under chronic treatment of the antidepressant imipramine. In addition, the expression of brain-derived neurotrophic factor (BDNF) and its downstream signaling was examined in the prefrontal cortex.
Results: Chronic exposure to imipramine reduced anxiety and sociability and problem-solving capacity, and increased thigmotaxis and day/night activity in all animals, but specifically in female SERT KO rats, compared to female wild-type (WT) rats. Further, we found an activation of BDNF-TrkB-Akt pathway signaling in the infralimbic, but not prelimbic, cortex after chronic imipramine treatment in SERT KO, but not WT, rats.
Limitations: Repeated testing behaviors could potentially affect the results. Additionally, the imipramine induced changes in behavior and in the BDNF system were measured in separate animals.
Conclusions: Our study indicates that female SERT KO rats, which mirror the female BD patients with the 5-HTTLPR s-allele, are at higher risk of a switch to mania-like behaviors under imipramine treatment. Activation of the BDNF-TrkB-Akt pathway in the infralimbic cortex might contribute to this phenotype, but causal evidence remains to be provided.

1. Introduction

Bipolar disorder (BD) is a severe mental disease with a lifetime prevalence of 1% (Pini et al., 2005). It is identified by the manifestation of mania or hypomania with overactivity, disinhibited behavior, and elation, interspersed with depression characterized by profound loss of motivation and interest (American Psychiatric Association, 2013). The impact of BD on patients can be devastating, with up to 15 % of patients committing suicide (Medici et al., 2015).

To date, although the etiology of BD is not fully known, neuroimaging studies have shown both structural and functional brain changes in patients (Merikangas et al., 2007). fMRI analysis found that in patients with BD, the psychosocial functioning is correlated with the activation of prefrontal cortex, which is an important region known to

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modulate human cognition and emotion and connected to the limbic brain areas (Townsend and Altshuler, 2012; Yoshimura et al., 2014; Fuster, 2001). With respect to the molecular mechanisms, alterations in brain-derived neurotrophic factor (BDNF) seem to be part of the BD's physiopathology (Wu et al., 2014; Scola and Andreazza, 2015). BDNF is enriched in brain regions such as cerebral cortex, involved in learning and memory (Lin and Huang, 2020; Caffino et al., 2020a), and acts as an important neurotrophin to regulate the survival and plasticity of neurons (Lee et al., 2021). In the clinic, serum levels of BDNF decrease in BD patients during manic and depressive episodes and increase in the case of recovery (Dunham et al., 2009).

While lithium is the standard drug for BD treatment (Won and Kim, 2017), other drugs are also used including antidepressants (e.g., imipramine), depending on the clinical picture (McCormick et al., 2015). Antidepressants constitute 50 % of the psychotropic drugs prescribed for BD (Antosik-Wójcińska et al., 2015). The improvement of clinical symptoms often manifests after a few weeks of drug treatment such as imipramine (Wu et al., 2014; Scola and Andreazza, 2015), presumably through the activation of various molecular pathways. One of these pathways involves the activation of BDNF and consequent phosphorylation of its high affinity receptor tyrosine receptor kinase B (TrkB) (Duman et al., 1997; Siuciak et al., 1997). Phosphorylation of TrkB finally activates the mitogen-activated protein kinase (MAPK)/extracellular regulated protein kinase (Erk) and phosphatidylinositol 3-kinase (P13k)/protein kinase B (Akt) signaling pathways, both of which modulate neuronal plasticity and protein translation (Slouzkey and Maroun, 2016; Hugues et al., 2006). Since dysregulation of Akt signaling has been implicated in psychiatric disorders (Duman and Voleti, 2012), we deem the BDNF-TrkB-Akt pathway in the prefrontal cortex of interest in the mechanistic understanding of antidepressant-mediated treatment of BD.

Though there are no clear sex differences in the prevalence of BD (Rubinow and Schmidt, 2019), sex differences are present in the subtypes, clinical signs and symptoms of features of this mood disorder (Swaab and Bao, 2020; Diflorio and Jones, 2010; Erol et al., 2015). It is also evident that there are sex differences in drug metabolism, which may relate to different effective doses of antidepressants between males and females (Kokras et al., 2011). A main concern in using antidepressants, specifically imipramine, is the risk of inducing an abrupt change in the mood from depression to mania called mania switch (Allain et al., 2017). This is observed in up to 40 % of patients (Koszewska and Rybakowski, 2009; Tondo et al., 2010), and often leads to hospitalization (Lim et al., 2005; Valentí et al., 2012). Females are more likely to experience rapid cycling and may be more susceptible to the antidepressant-induced switch to mania (Rubinow and Schmidt, 2019; Arnold, 2003).

From a genetic perspective, BD has a high genetic predisposition with a heritability index of 0.85 (McGuffin et al., 2003), which is observed with a higher genetic correlation in females (i.e., 0.48 in females, 0.04 in males) (Blokland et al., 2022). A meta-analysis showed that BD is strongly associated with the low activity short (s) allelic variant of the serotonin transporter (5-HTT)-linked polymorphic region (5-HTTLPR s-allele) (Frye et al., 2015). Genetic 5-HTT inactivation reduces the clearance of synaptic serotonin and increases extracellular serotonin levels, which possibly explains higher levels of serotonin's metabolite 5-hydroxyindoleacetic acid (5-HIAA) in the cerebrospinal fluid of BD patients (Pålsson et al., 2017). Of interest, the serotonin transporter knockout (SERT KO) rats exhibit increased extracellular serotonin but unaltered dopamine levels (Verheij et al., 2014), and demonstrate both depression-like (reduced sociability, anhedonia and behavioral despair) (Olivier et al., 2008; Homberg et al., 2007a) and mania-like (increased switching, slightly increased motivation and psychostimulant-induced hyperactivity) phenotypes (Nonkes et al., 2013; Homberg et al., 2008; Nonkes et al., 2014; Homberg et al., 2010). In addition, in response to an atypical antipsychotic (i.e., lurasidone) with antidepressant effects, SERT KO rats 'switch' from most to least conditioned freezing (Luoni et al., 2013; Fumagalli et al., 2012). SERT KO mice also remain sensitive to the antidepressant-like effects of imipramine (Holmes et al., 2002). SERT KO rats model the human 5-HHTLPR s-allele regarding stress sensitivity (Schipper et al., 2019). Therefore, the female SERT KO rat can possibly serve as a model to understand the neural mechanisms of BD associated with the 5-HTTLPR s-allele in female patients and the imipramine-induced switch to mania. Neurochemically, SERT KO rats display a reduction in BDNF in the hippocampus and prefrontal cortex starting from the second postnatal week for the rest of life (Sbrini et al., 2020; Calabrese et al., 2013; Guidotti et al., 2012).

To date, the mechanisms of the mania switch under imipramine treatment, particularly in female patients, are unclear. The current study was set out to recapitulate the clinical symptoms of the switch to mania under imipramine treatment using the female SERT KO rat model and to examine the potential underlying mechanism by focusing on the BDNF-TrkB-Akt pathway in two subregions of the prefrontal cortex, prelimbic and infralimbic cortices. We hypothesized that, under imipramine treatment, female SERT KO rats have a higher risk for a switch from depression-like to mania-like behaviors through an increased activity of the BDNF-TrkB-Akt pathway, compared to wild-type (WT) rats.

To test our hypothesis, female SERT KO rats were subjected to a behavioral test battery measuring depression- and mania-like behaviors under control conditions and after chronic imipramine treatment. Molecular changes in the BDNF-TrkB-Akt pathway in the prefrontal cortex were also investigated. Results showed that chronic imipramine treatment increased signs of mania-like behavior, reduced problem-solving abilities, altered home-cage activity, and induced prefrontal cortex changes in BDNF-TrkB-Akt signaling, which were more pronounced in SERT KO rats compared to WT rats.

2. Materials and methods

2.1. Animals

A total of 54 female WT and SERT KO rats at the age of 70 days (weighting 250-300 g) were used for our studies. Additionally, 11 WT and 11 SERT KO male rats at the age of 70 days were subjected to the behavioral tests (see Supplementary Figs. 1–4 and Supplementary Table 1). The SERT KO rats (Slc6a41Hubr) have been generated by target-selected ENU-induced mutagenesis and been outcrossed for at least 20 generations with commercial Wistar rats. The genotyping information has been published previously (Homberg et al., 2007b). The female estrous cycle was not checked and controlled in these animals. The sample size ($N = 11$) was a priori determined by a Power analysis (effect size = 0.32, correlation between repeated measures = 0.5, $\alpha = 0.05$, correlation between repeated measures = 0.5, $1\text{-}\beta = 0.8$, number of measurements = 2) and checked by a biostatistician. The animals' genotypes were blind to the researcher who conducted the behavioral experiments. Animals were socially housed in groups of two and maintained at temperature of 21–22 °C ± 1 °C and under a 12 h light/dark cycle (8:00 on, 20:00 off). They had free access to food and water ad libitum. Rats were gently handled once daily for a week prior to the behavioral tests. The experimental procedures were performed under a project license from the Central Committee on Animal Experiments (Centrale Commissie Dierproeven, The Hague, The Netherlands), in full compliance with the legal requirements of Dutch legislation on the use and protection of laboratory animals (Animal Testing Act). All efforts were made to reduce the number of animals used and their suffering.

2.2. Drugs

Pure imipramine powder, purchased from the Radboud University Medical Center pharmacy, was dissolved in water and administered orally through the drinking water. We have chosen this delivery method because it alleviates the animals from repeated injection stress, which

could confound the results. Because water with imipramine caused animals to drink less, 2 % sucrose was added to the drinking water of all animals (i.e., with or without imipramine), eliminating the influence of imipramine taste. The volume of left water in the bottle was measured every day. One rat drank around 20 ml water per day and each rat was estimated to consume 30 mg/kg imipramine per day.

2.3. Behavioral testing and treatment procedure

To assess depression-like and mania-like behaviors, 11 WT and 11 SERT KO female, along with 11 WT and 11 SERT KO male rats (Supplementary Figs. 1–4 and Supplementary Table 1) underwent a test battery (Fig. 1), which was conducted twice, first at baseline and subsequently after imipramine treatment. The animals were provided 2 % sucrose water for four weeks during the first set of behavioral tests for the baseline condition, and sequentially were delivered imipramine for a total of six weeks for the treatment condition (i.e., two weeks before the tests, four weeks during the second set of behavioral tests). One hour before testing, animals were placed in the test room in their home cage to acclimatize to the new environment. The test room was illuminated by white light at 12 Lux. After each behavioral test, rats were returned to their home cages.

2.4. Elevated plus maze test

The elevated plus maze test was conducted to assess anxiety-related behavior (Olivier et al., 2008; Hogg, 1996; Homberg et al., 2011). The test device was elevated to a height of 50 cm with two open arms (50 cm length × 10 cm width) and two enclosed arms (50 cm length × 10 cm width × 40 cm height) with a square central cross connection area (10

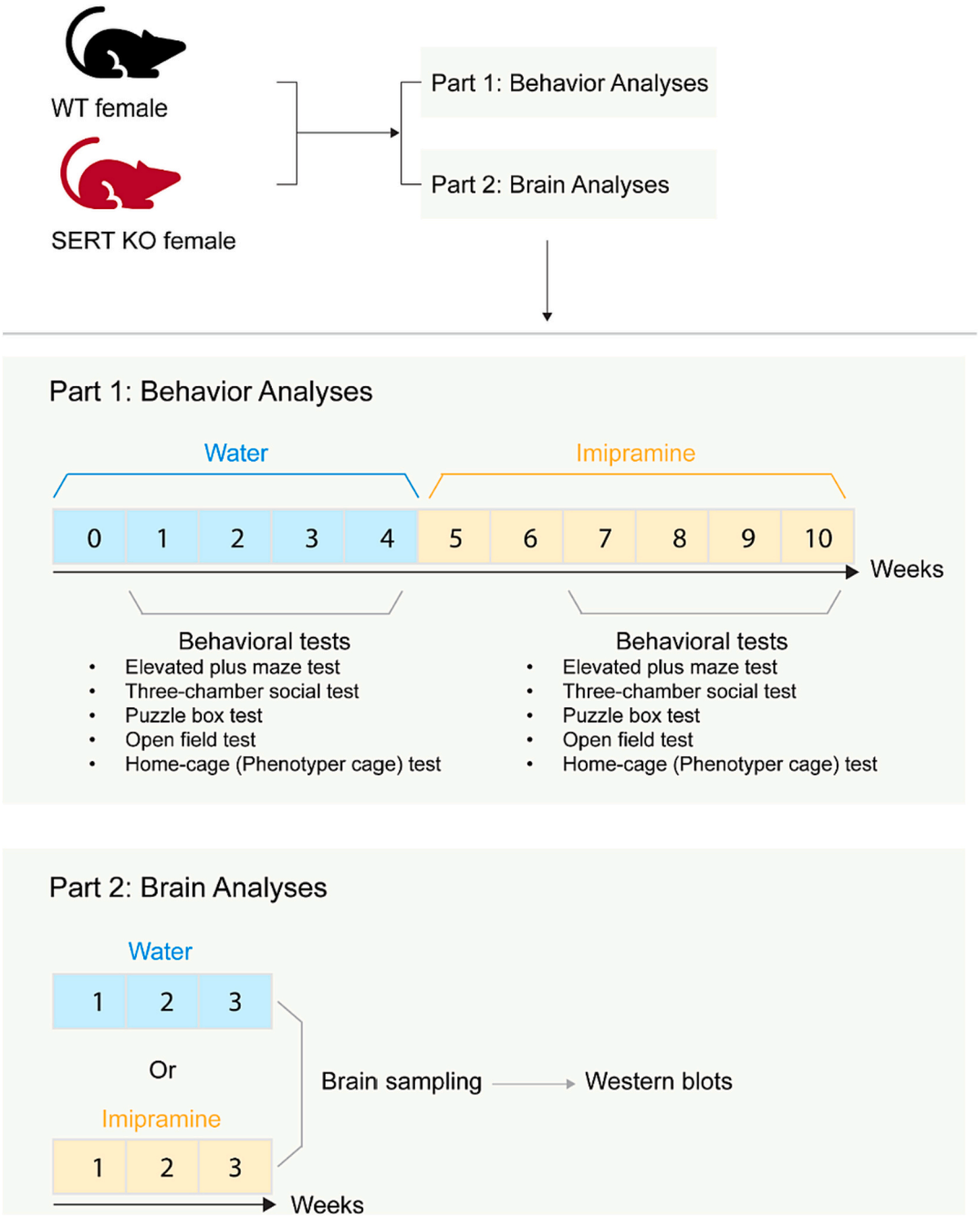

Fig. 1. The chart flow of the experimental procedure.

cm × 10 cm). The animal was placed in the center of the maze, facing one of the open arms, for a free exploration period of 5 min. The movements and position of the animals were automatically recorded and measured using EthoVision XT 10 software (Noldus Information Technology, Wageningen, The Netherlands). Results are expressed as the mean of time spent (s) in open arms, the mean time spent in closed arms, and the distance moving (cm) in both open and closed arms.

2.5. Three-chamber social novelty test

To investigate the sociability and social novelty, the three-chamber social novelty test was performed using previous protocols (Manfré et al., 2018; Crews et al., 2012). The test apparatus was a rectangular cage separated into three chambers with the same size (30 cm length× 35 cm width × 35 cm height). Through small openings, two lateral chambers were connected to the central one. The small chambers contained wire cages. Rats can easily move through these three chambers. The test consisted of three consecutive trials: habituation (5 min), social interaction (10 min, 1 stranger rat present), and social novelty (10 min, 2 stranger rats present). There was a 7 min interval between test trials. In the first trial (habituation), an experimental rat was placed in the central chamber and was allowed to explore all chambers and habituate for 5 min. In the second trial (social interaction), a stranger rat was introduced to the wire cage in one of the lateral chambers, while the experimental rat was placed in the center chamber and allowed to explore all chambers for 10 min. In the third trial (social novelty), a second stranger rat was placed in the wire cage of the opposite lateral chamber. The experimental rat could choose whether to explore the first stranger rat that was already present trial 2 (familiar rat) or the newly introduced one (novel rat). The behavior was video-taped and the time the experimental rats spent on exploring each chamber and the wire cages was measured using Ethovision software. The two stranger rats had the same age, sex, and genotype as the experimental rat.

2.6. Puzzle box test

The puzzle box test was applied to determine a rat's native problem-solving ability (Ben Abdallah et al., 2011; Galsworthy et al., 2005). The test device consisted of two compartments (i.e., a brightly open-field box of 75 cm in length × 28 cm in width × 25 cm in height and a sheltered dark goal-box of 15 cm in length× 28 cm in width × 25 cm in height). The two boxes were connected through a small door with a 4 cm width. A rat was placed in the center of the open-field box, from which it could easily go into the dark goal-box through the small door, avoiding the light. If the rat could not enter the goal-box within 180 s, a failure was recorded. Each rat performed three trials per day for three consecutive days of testing with three obstruction conditions. The third trial of a given obstruction condition was always conducted on a subsequent day (see Table 1). The experimental videos were recorded using Ethovision software. The time the rats needed to enter the goal-box with four paws was manually measured as the problem-solving latency.

Table 1
Scheme of the Puzzle box test. The Puzzle box test was run for three days, and consisted of three obstruction conditions.

Day	Condition	Trial	Obstruction	Time limit (s)
1	0	1	Open door with no obstructions	180
	1	2	Open channel within doorway	180
	1	3	Open channel within doorway	180
2	1	4	Open channel within doorway	180
	2	5	Channel filled with sawdust	180
	2	6	Channel filled with sawdust	180
3	2	7	Channel filled with sawdust	180
	3	8	Channel blocked with tissue	180
	3	9	Channel blocked with tissue	180

2.7. Open field test

Locomotor activity and anxiety-like behavior were evaluated using the open field test (Peeters et al., 2018; Golebiowska et al., 2019; Bosch et al., 2022). The experiments were conducted in an open field arena (50 cm length × 50 cm width × 50 cm height). The floor of the arena was defined as sixteen squares, in which the four middle squares were designated as the center. Rats were put in the center and allowed to freely move throughout the arena for 1 h. The total distance moved, velocity, and time spent in the center were tracked by Ethovision XT10 software (Noldus Information Technology, the Netherlands). Under the condition of imipramine treatment, data of 6 WT and 7 SERT KO rats were lost due to the technical issue of video noise.

2.8. Home-cage activity

To detect the anxiety under a relatively natural environment preventing any influence from stress, locomotor activity was monitored in an automated home-cage (Grieco et al., 2021; Klein et al., 2022; Kyriakou et al., 2018). Rats were housed individually in a plexiglass Phenotyper cage (45 cm in length × 45 cm in width × 55 cm in height) (Noldus Information Technology, the Netherlands) for a total duration of three consecutive days. Each device had a water bottle, a feeding station and a shelter (14.3 cm × 14.3 cm × 11.5 cm) in one corner. Food and water were provided ad libitum. The behavior of the animal was recorded with an infrared camera on the top of the cage. The total moving distance in the light and dark phases were measured using EthoVision 3.1 software (Noldus Information Technology, the Netherlands). No human interference took place between the start and the end of the observations. Because of the technical issue of video noise, the following data were lost: 1 WT rat on day 1 of the light phase under the water condition; 2 WT and 2 SERT KO rats on day 1–3 of the light phase under imipramine treatment; 1 SERT KO rat on day 2 of the dark phase under the water condition; 1 WT rat on day 1, 2 WT rats on day 2, 3 WT rats on day 3, 1 SERT KO rat on day 1, and 2 SERT KO rats on day 2–3 of the dark phase under imipramine treatment.

2.9. Western blot analyses

Protein expression levels were investigated in female WT ($n = 6$) and SERT KO (n = 6) rats. We focused on the females only, because females show a more pronounced shift to mania-like behavior (specifically hyperactivity) compared to males (see supplementary figs. 1–4 and results section). Half rats in each group were given 2 % sucrose water and the other half was given 2 % sucrose plus imipramine via the drinking water for 21 days. These animals did not undergo behavioral tests to avoid the potential influence of repeated behavioral testing on protein expression. Twenty-four hours after the last treatment day, the animals were decapitated without anesthesia. Brains were removed and stored at −80 °C.

Using the rat brain atlas (Paxinos and Watson, 2007), the prelimbic and infralimbic cortices (coordinates between bregma +4.20 mm and bregma +2.52 mm) were punched from frozen brain sections of 220 μm using a sterile 1-mm-diameter needle (Giannotti et al., 2016). Punches from the right and left hemisphere were pooled. Prelimbic and infralimbic tissue were stored at −80 °C until being processed for molecular analysis.

Proteins from prelimbic and infralimbic cortices were homogenized in a glass-glass potter in cold 0.32 M sucrose buffer pH 7.4 containing 1 mM HEPES, 0.1 mM PMSF, in presence of commercial cocktails of protease (cOmplete™ Protease Inhibitor Cocktail, Roche, Monza, Italy) and phosphatase (Sigma–Aldrich, Milan, Italy) inhibitors and then sonicated (Caffino et al., 2021). Total proteins were measured in the whole homogenate and quantified according to the Bradford Protein Assay procedure (Bio-Rad, Milan, Italy), using bovine serum albumin as calibration standard, and stored at −20 °C for subsequent molecular

analysis. Western blots (WB) were run using sodium dodecyl sulfate - 14 % polyacrylamide gels under reducing conditions as previously described (Caffino et al., 2017) on the whole homogenate lysate (10 μg) of infralimbic and prelimbic cortices and then electrophoretically transferred (wet transfer) onto nitrocellulose membranes (GE Healthcare). The strips of nitrocellulose membrane close to the molecular weights at which the bands of the protein of interest were expected were cut from the entire squared blot (full areas) as suggested by their specific molecular weight and the information present in the datasheet of the antibody. Blots were blocked for 1 h at room temperature (25 ± 2 °C) with I-Block solution (Life Technologies Italia) in TBS + 0.1 % Tween-20 buffer and washed with TBS + 0.1 % Tween-20 buffer. The conditions of the primary antibodies were the following: anti mBDNF (1:500, Icosagen, cod: 327–100, RRID: AB_2927780), anti phospho-TrkB Tyr706 (1:200, Novus Biologicals, cod: NBP2–54764), anti phospho-Akt Ser473 (1:1000, Cell Signaling Technology, cod: 9271), anti-total TrkB (1:1000, Cell Signaling Technology, cod: 4603, RRID: AB_2155125), Akt (1:1000, Cell Signaling Technology, cod: 9272) and anti β-actin (1:5000, Sigma-Aldrich, cod: A5441, RRID: AB_476744). Results were standardized to β-actin control protein, which was detected by evaluating the band

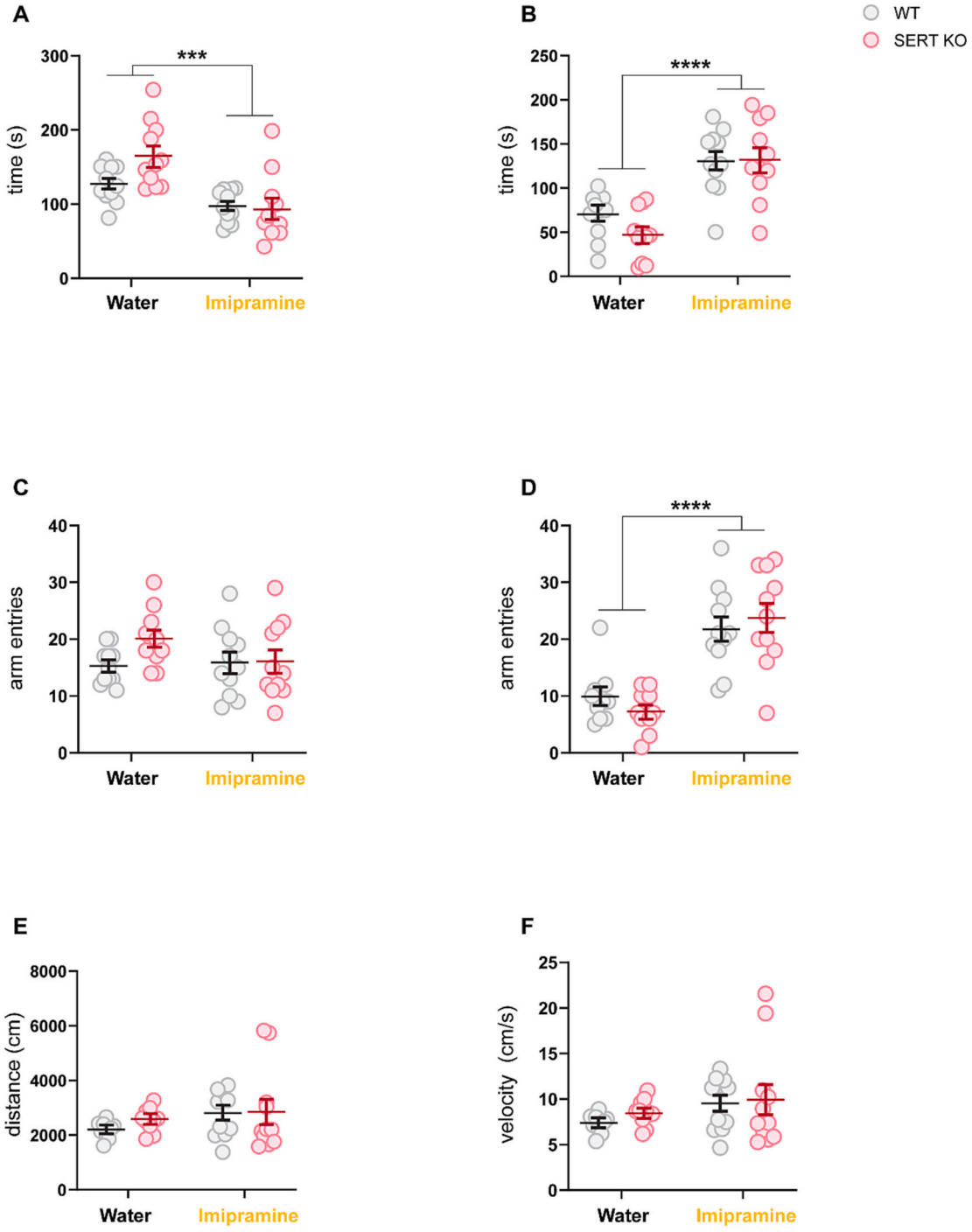

Fig. 2. Anxiety-like behavior of female WT and SERT KO rats assessed using the elevated plus maze test. (A) Time spent in closed arms. (B) Time spent in the open arms. (C) The frequency of entering closed arms. (D) The frequency of entering open arms. (E) Total distance traveled. (F) Average velocity. Each circle represents an individual rat. The horizontal bar and error bars represent mean ± S.E.M. ***$P < 0.001$ and ****$P < 0.0001$ indicate a significant effect of treatment (Mixed model, $n = 11$ per group). WT = wild-type rats; SERT KO = serotonin transporter knock-out rats.

density at 43 kDa. Immunocomplexes were visualized by chemiluminescence using the Chemidoc MP Imaging System (Bio-Rad Laboratories, RRID: SCR_019037) after 2–3 min of enhanced chemiluminescence substrate (ECL) exposure (Cyanagen Srl), and subsequently analyzed with Image LabTM software (Bio-Rad, RRID: SCR_014210). Examples of full-size original and merged cropped immunoblots are presented in the supplementary figs. (S1-S2, respectively). Activation of the proteins investigated was expressed as a ratio between the phosphorylated and the respective total forms and analyzed. Gels were run two times each, and the results represent the average from two different runs. We used a correction factor to average the different gels: correction factor gel B = average of (OD protein of interest/OD b-actin for each sample loaded in gel A)/(OD protein of interest/OD β-actin for the same sample loaded in gel B) (Caffino et al., 2020b).

2.10. Statistical analysis

Statistical analysis was done using IBM SPSS Statistics for Windows, Version 27.0. Data were checked for normality. Because normality was for some datasets violated, all results were analyzed using a Linear mixed model with genotype and treatment as between-subjects factors. The Sidak test or Tukey test was adopted for multiple post-hoc comparisons if the interaction between genotype and treatment was significant ($P < 0.05$). NS = non-significant. Data were collected in individual animals (independent determinations) and are presented as mean ± standard error of the mean for each group.

3. Results

3.1. Chronic imipramine treatment reduces anxiety-like behavior in WT and SERT KO rats in the elevated plus maze test

The elevated plus maze was applied to evaluate the anxiety behavior in WT and SERT KO rats. Under the imipramine treatment, female rats spent more time in the open arms and less time in the closed arms. Mixed model analysis revealed a significant treatment effect on time spent in the closed arms ($F_{(1,40)} = 22.897$, $P < 0.001$) but there was no significant genotype ($F_{(1,40)} = 2.485$, P═NS) and interaction of genotype × treatment ($F_{(1,40)} = 3.936$, P═NS) effect (Fig. 2A). For time spent in the open arms, analysis revealed a significant treatment effect ($F_{(1,40)} = 47.760$, $P < 0.0001$) (Fig. 2B). We did not observe a significant genotype effect ($F_{(1,40)} = 0.986$, P═NS) nor a significant genotype × treatment interaction effect ($F_{(1,40)} = 1.354$, P═NS). Regarding the frequency of entering closed arms, no significant differences were seen (genotype effect ($F_{(1,40)} = 2.394$, P═NS), treatment effect ($F_{(1,40)} = 1.083$, P═NS), genotype × treatment interaction effect ($F_{(1,40)} = 2.058$, P═NS)), but a significant treatment effect was found for open arm entries ($F_{(1,40)} = 55.300$, $P < 0.0001$) without significant genotype ($F_{(1,40)} = 0.021$, P═NS) and interaction ($F_{(1,40)} = 1.439$, P═NS) effects. As shown in Fig. 2C-2D, the number of open arm entries was increased in imipramine treated rats independent of genotype. Finally, no statistical significance was found for total distance moved (treatment effect ($F_{(1,40)} = 2.575$, P═NS; genotype effect ($F_{(1,40)} = 0.530$, P═NS); genotype × treatment interaction effect ($F_{(1,40)} = 0.303$, P═NS)) and average velocity (treatment effect ($F_{(1,40)} = 3.306$, P═NS; genotype effect ($F_{(1,40)} = 0.640$, P═NS); genotype × treatment interaction effect ($F_{(1,40)} = 0.158$, P═NS)) (Fig. 2E-2F). Male rat behavior was also influenced by imipramine treatment, with imipramine treated WT and SERT KO rats spending less time in the closed arms and more time in the open arms and entering closed arms less frequently (Supplementary Fig. 1). These results show that under chronic imipramine treatment, all rats, regardless of genotype and sex, significantly spent less time in the closed arms and more time and entries in the open arms.

3.2. Chronic imipramine treatment reduces sociability and social novelty seeking preference in SERT KO and WT rats in the three-chamber box

To test the effect of imipramine on rats' sociability, we applied the three-chamber box test. Under imipramine treatment, female WT and SERT KO rats spent less time in sniffing a stranger rat. A mixed model revealed a significant treatment effect in the sniffing time of a stranger rat ($F_{(1,40)} = 15.543$, $P < 0.0001$), while no significant genotype ($F_{(1,40)} = 0.868$, P═NS) and genotype × treatment interaction ($F_{(1,40)} = 1.305$, P═NS) effects were found (Fig. 3A). As shown in Fig. 3B, sniffing the familiar rat was significantly impacted by treatment ($F_{(1,40)} = 5.142$, $P < 0.05$), but no significances were observed in genotype ($F_{(1,40)} = 0.914$, P═NS) and interaction of genotype × treatment ($F_{(1,40)} = 0.080$, P═NS). Similarly, sniffing novel rat was significantly influenced by treatment ($F_{(1,40)} = 6.509$, $P < 0.05$), but not by genotype ($F_{(1,40)} = 1.295$, P═NS). There was also no genotype × treatment effect for this parameter ($F_{(1,40)} = 1.410$, P═NS). These data showed that female rats displayed reduced sociability and social novelty under the imipramine treatment. This phenotype was not significant between WT and SERT KO female rats. In male rats we found a similar phenotype as female rats; under imipramine treatment the male rats spent less time on sniffing a stranger and a novel rat. Yet, a genotype effect was seen for the male rats, as male SERT KO rats spent more time on sniffing the familiar rat in each condition (Supplementary Fig. 2). These data demonstrate that sociability and social novelty were reduced by imipramine treatment in both female and male rats.

3.3. Chronic imipramine treatment reduces problem-solving abilities in SERT KO rats

To assess the abilities of problem-solving impacted by the imipramine treatment, the puzzle box test was performed. In general, female rats needed more time to solve the problem under imipramine treatment. This phenotype is significant in SERT KO rats. Statistics revealed a significant treatment and genotype effect on puzzle box performance ($F_{(1,379)} = 3.991$, $P < 0.05$, $F_{(1,379)} = 5.277$, $P < 0.05$, respectively). In addition, there was a significant genotype × treatment interaction effect for the latency to reach the goal box ($F_{(1,379)} = 4.652$, $P < 0.05$) (Fig. 4). However, Sidak test post-hoc testing did not detect statistical differences between groups for any trial.

Interestingly, the significant treatment and genotype effects on problem-solving abilities were also observed in male rats, but there was no treatment x genotype interaction effect. (Supplementary Fig. 3). These data showed that imipramine treatment altered problem-solving behavior in all animals, particularly in female SERT KO rats (i.e., in the female rats a treatment x genotype effect was found).

3.4. Chronic imipramine treatment decreases center time in female SERT KO and WT rats in the open field test

The open field test was applied to test the rats' general activity and exploratory behavior under imipramine treatment. The total distance moved (i.e., (novelty-induced) exploratory behavior) and time spent in the center area (i.e., thigmotaxis) of the open field test were analyzed. As shown in Fig. 5A and B, we found a significant treatment effect on total distance moved in the first (novelty conditions), but not in the second 30 min after exposure to the open field box ($F_{(1,27)} = 9.201$, $P < 0.01$; $F_{(1,27)} = 2.355$, P═NS, respectively). No significant genotype (first 30 min: $F_{(1,27)} = 0.099$, P═NS, second 30 min: $F_{(1,27)} = 1.835$, P═NS) and genotype × treatment interaction effect was observed in the first and second 30 min ($F_{(1,27)} = 0.214$, P═NS, $F_{(1,27)} = 0.255$, P═NS, respectively) (Fig. 5A and B). Thus, novelty-induced exploratory behavior decreased significantly in both female WT and SERT KO rats under imipramine treatment.

Regarding the time spent in the center, a significant treatment effect was found for the first 30 min ($F_{(1,27)} = 17.460$, $P < 0.0001$), whereas no

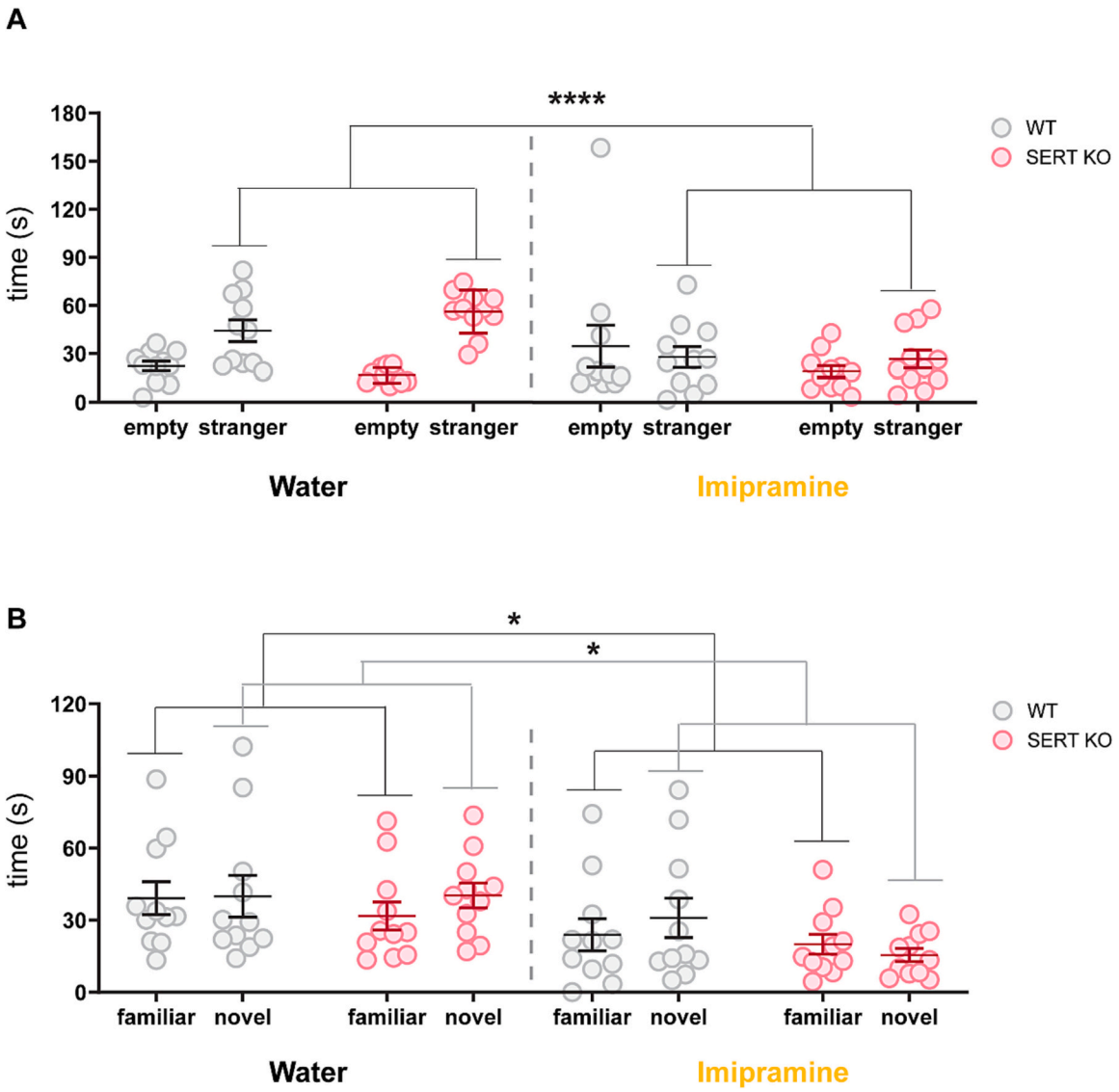

Fig. 3. Sociability and social novelty seeking behavior in female WT and SERT KO rats assessed using the three-chamber test. (A) Time spent on sniffing the empty cage and stranger rat. (B) Time spent on sniffing the familiar and novel rats. Each circle represents an individual rat. The horizontal bar and error bars represent mean ± S.E.M. *$P < 0.05$ and ****$P < 0.0001$ indicate a significant effect of treatment (Mixed model, $n = 11$ per group). WT = wild-type rats; SERT KO = serotonin transporter knock-out rats.

significant genotype ($F_{(1,27)} = 1.825$, P=NS) and genotype × treatment ($F_{(1,27)} = 0.155$, P=NS) effects were observed (Fig. 5C). In the second 30 min exposure to the test, a genotype effect on time spent in the center was found ($F_{(1,27)} = 5.927$, $P < 0.05$). However, there was no significant treatment ($F_{(1,27)} = 2.811$, P=NS) and genotype × treatment interaction ($F_{(1,27)} = 1.512$, P=NS) effect in this period (Fig. 5D). These data indicate that female rats show increased thigmotaxis under imipramine treatment, and that female SERT KO rats displayed a preference for the wall area (i.e., less time in the central area).

Interestingly, we did not observe any significant effects of genotype, treatment, and their interaction in the male rats (Supplementary Fig. 4). In conclusion, female rats were more sensitive to imipramine treatment in the open field test than the male rats and showed a decrease in exploratory behavior and increase in thigmotaxis.

3.5. *Chronic imipramine treatment increases locomotor activity of female SERT KO rats during the light phase in the homecage*

To monitor daily activity over 72 h, rats were housed in the homecage (Phenotyper). The female rats became more active under imipramine treatment during both the light and dark phase, particularly the SERT KO rats. The data revealed a significant effect of treatment and treatment × genotype interaction on total distance moved during the light phase ($F_{(1,116)} = 8.177$, $P < 0,01$; $F_{(1,116)} = 5.392$, $P < 0.05$, respectively). No genotype effect was found ($F_{(1,116)} = 3.516$, P=NS). Sidak post hoc testing revealed that after treatment with chronic imipramine, distance moved was significantly increased in SERT KO rats as compared to WT rats (light phase day 2 and 3) ($P < 0.05$) (Fig. 6A). During the dark phase, significant treatment effects were identified ($F_{(1,127)} = 13.201$, $P < 0.0001$), but there was no significant genotype effect ($F_{(1,127)} = 2.008$, P=NS) and treatment × genotype interaction effect ($F_{(1,127)} = 1.196$, P=NS) (Fig. 6B). Moreover, imipramine increased the rats' velocity during the light phase with a significant treatment effect ($F_{(1,124)} = 6.472$, $P < 0.05$). There was no genotype ($F_{(1,124)} = 1.471$, P=NS) and treatment × genotype interaction ($F_{(1,124)} = 0.770$, P=NS) effect (Fig. 6C). No statistical significance was found for velocity during the dark phase (treatment effect ($F_{(1,125)} = 2.243$, P=NS), genotype effect ($F_{(1,125)} = 1.705$, P=NS), treatment × genotype

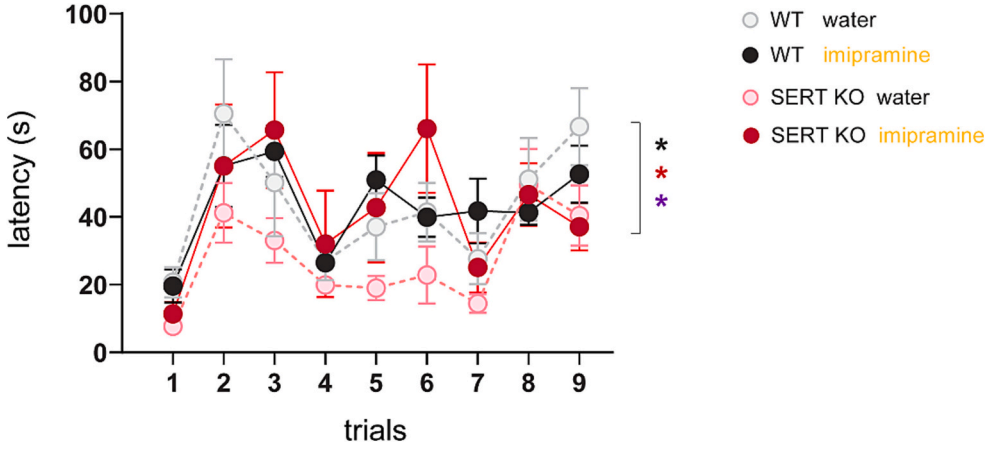

Fig. 4. Problem-solving abilities of WT and SERT KO rats assessed by puzzle box test. The y-axis shows the latency to reach the goal box, the x-axis represents the number of trials. Each circle represents one group in the position of mean. The error bars represent the mean ± S.E.M. *P < 0.05, and *P < 0.05, and *P < 0.05 indicate a significant effect of treatment, genotype, and interaction of treatment × genotype, respectively (Mixed model, $n = 11$ per group). WT = wild-type rats; SERT KO = serotonin transporter knock-out rats.

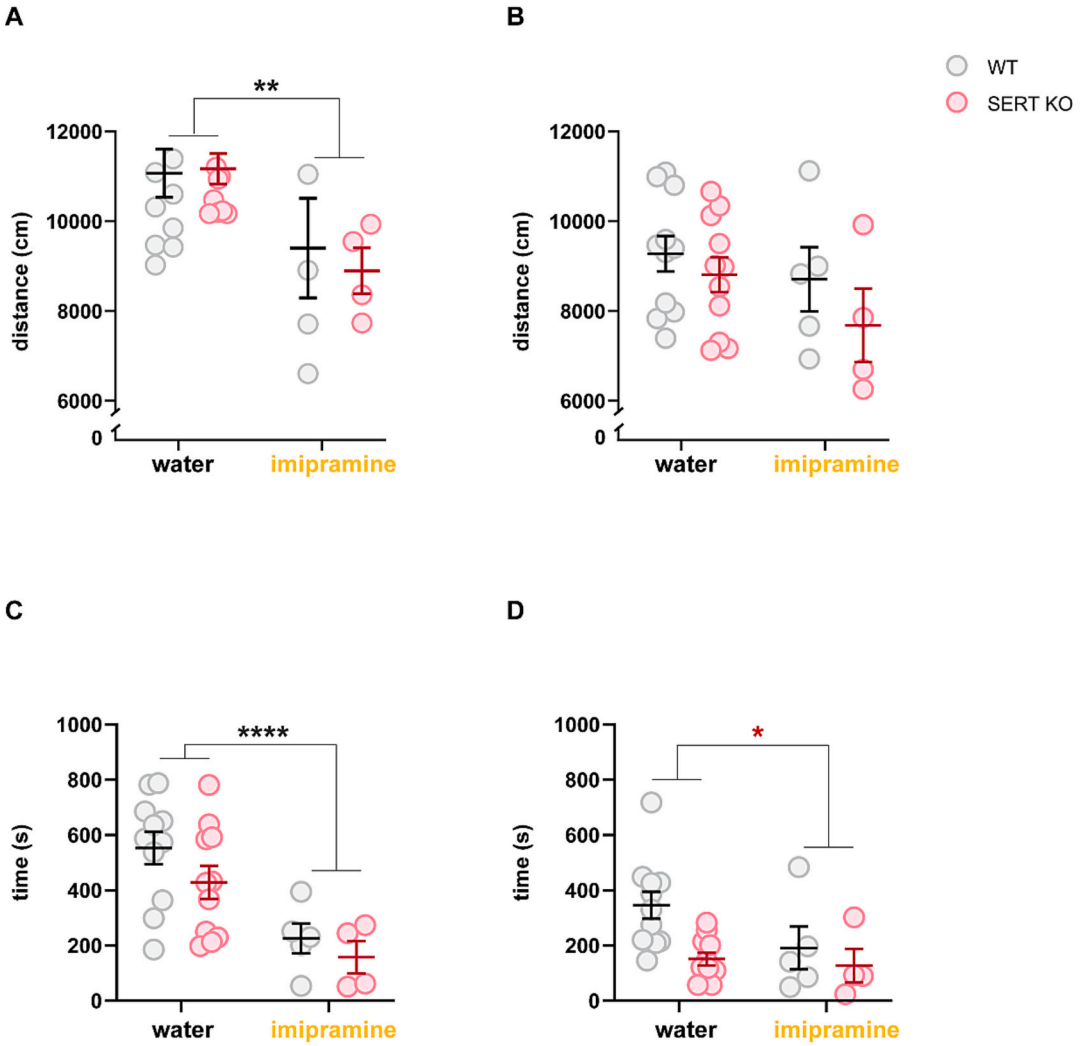

Fig. 5. Exploratory and anxiety-like behavior of WT and SERT KO rats assessed using the open field test. (A) Total distance moved during the first 30 min after exposure to the open field box. (B) Total distance moved during the second 30 min after exposure to the open field box. (C) Time spent in the central area during the first 30 min after exposure to the open field box. (D) Time spent in the central area during the second 30 min after exposure to the open field box. Each circle represents an individual rat. The horizontal bar and error bars represent the mean ± S.E.M. **$P < 0.01$ and ****$P < 0.0001$ indicate a significant effect of treatment, *$P < 0.05$ indicates a significant effect of genotype (mixed model, $n = 11$ per group in the water condition; WT $n = 5$ and SERT KO $n = 4$ in the imipramine treatment condition). WT = wild-type rats; SERT KO = serotonin transporter knock-out rats.

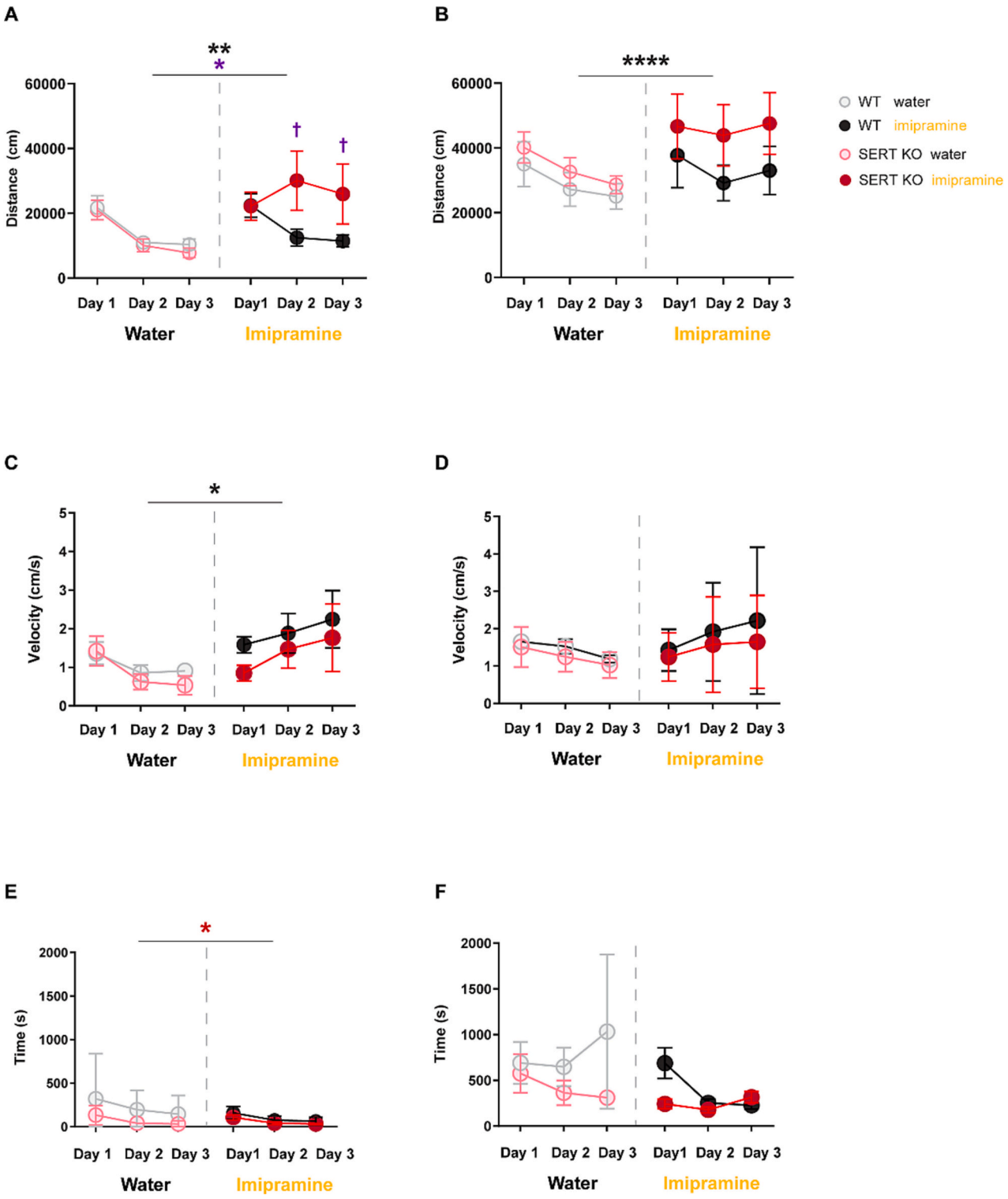

Fig. 6. Locomotor activity of WT and SERT KO rats assessed in the home-cage test. (A) The total distance moved in the light phase. (B) The total distance moved in the dark phase. (C) The average velocity in the light phase. (D) The average velocity in the dark phase. (E) Time spent in the center in the light phase. (F) Time spent in the center in the dark phase. Each circle represents one group in the position of mean. The error bars represent mean ± S.E.M. *$P < 0.05$, **$P < 0.01$, and ****$P < 0.0001$ indicate a significant effect of treatment; *P < 0.05 indicates a significant effect of genotype; *P < 0.05 indicate a significant effect of interaction of treatment × genotype (Mixed model). †P < 0.05 indicate a significant difference between groups at the same day (Sidak post hoc test). WT = wild-type rats; SERT KO = serotonin transporter knock-out rats.

interaction effect ($F_{(1,125)} = 0.610$, P=NS)) (Fig. 6D). Finally, the time rats spent in the central area was analyzed (Fig. 6E-6F). During the light phase, SERT KO rats spent less time in the central area. Statistical analysis revealed a significant genotype effect ($F_{(1,80)} = 5.417$, $P < 0.05$), in the absence of treatment ($F_{(1,80)} = 1.022$, P=NS) and treatment × genotype interaction ($F_{(1,80)} = 1.022$, P=NS) effects. During the dark phase, there were no effects of treatment, genotype and their interaction on time spent in the central area (treatment effect: $F_{(1,87)} = 0.282$,

P=NS; genotype effect: $F_{(1,87)} = 1.405$, P=NS; treatment × genotype interaction effect; $F_{(1,87)} = 0.282$, P=NS). The data demonstrate that imipramine treatment increased homecage activity in both SERT KO and WT rats, during both the light and dark phase, particularly in SERT KO rats during the light phase. Data from male rats are lacking due to technical issues.

3.6. Chronic imipramine treatment modulates BDNF signaling in female SERT KO rats

We first evaluated the expression of mature BDNF (mBDNF) in the whole homogenate of rat prelimbic or infralimbic cortices in water- and imipramine-treated WT or SERT KO rats. In the prelimbic cortex (Fig. 7A-B, S Fig. 1A), mixed model analysis revealed a genotype × treatment interaction effect ($F_{(1,20)} = 36.339$, $P < 0.0001$), but no significant genotype ($F_{(1,20)} = 0.335$, P=NS) and treatment ($F_{(1,20)} = 0.498$, P=NS) effect (Fig. 7A). Tukey's multiple comparisons indicated that mBDNF expression was significantly reduced in SERT KO rats at baseline ($P < 0.01$), whereas imipramine administration significantly diminished mBDNF expression in WT rats and increased it in SERT KO rats ($P < 0.001$). In the infralimbic cortex (Fig. 7C-D, S Fig. 1B), significant effects of genotype ($F_{(1,20)} = 10.533$, $P < 0.01$), treatment ($F_{(1,20)} = 12.101$, $P < 0.01$), and a genotype × treatment interaction were detected ($F_{(1,20)} = 5.566$, $P < 0.05$) (Fig. 7C). Following the post hoc comparisons, we found that mBDNF expression was significantly increased in SERT KO rats at baseline ($P < 0.01$), whereas imipramine administration significantly increased mBDNF expression in WT rats ($P < 0.01$), while leaving it unaltered in SERT KO rats (P=NS).

We then moved to the high-affinity receptor of BDNF, i.e., TrkB, as an index of activation of BDNF downstream signaling. We measured the ratio between the phosphorylated form of TrkB and its total levels, i.e., pTrkB/TrkB. In the prelimbic cortex (Fig. 7E-F, S Fig. 1A), mixed model analysis revealed no significant effects of genotype ($F_{(1,20)} = 0.119$, P=NS), treatment ($F_{(1,20)} = 0.147$, P=NS), and the genotype x treatment interaction ($F_{(1,20)} = 0.344$, P=NS) (Fig. 7E). In the infralimbic cortex (Fig. 7G-H, S Fig. 1B), no significant effect of genotype ($F_{(1,20)} = 1.865$, P=NS) was found, while the effects of treatment ($F_{(1,20)} = 15.980$, $P < 0.001$) and genotype × treatment interaction were significant ($F_{(1,20)} = 11.016$, $P < 0.01$) (Fig. 7G). Post hoc comparisons by Tukey's test showed that pTrkB/TrkB was significantly increased in SERT KO rats, compared to WT rats, after imipramine treatment ($P < 0.05$). Finally, we investigated the activation of the Akt pathway, which represents one of the main pathways downstream of BDNF. As an index of activation of this pathway, we measured the ratio between the phosphorylated form of Akt and its total levels, i.e., pAkt/Akt. In the prelimbic cortex (Fig. 7I-J, Supplementary Fig. 5 A), we observed significant effects of genotype ($F_{(1,20)} = 18.414$, $P < 0.001$) and treatment ($F_{(1,20)} = 6.084$, $P < 0.05$), but no significant interaction effect ($F_{(1,20)} = 0.016$, P=NS) (Fig. 7I). In the infralimbic cortex (Fig. 7K-L, Supplementary Fig. 5B), no significant effects of genotype ($F_{(1,20)} = 0.778$, P=NS) and treatment ($F_{(1,20)} = 0.089$, P=NS) were seen, while we detected a significant genotype × treatment interaction ($F_{(1,20)} = 36.496$, $P < 0.0001$) (Fig. 7K). Post-hoc testing indicated a significant lower expression of pAkt/Akt in SERT KO rats ($P < 0.01$) at baseline, whereas imipramine treatment significantly increased it in SERT KO rats, compared to WT rats ($P < 0.001$). These results demonstrate that the expression of mBDNF and its downstream effectors TrkB and Akt are consistently increased in the infralimbic cortex of SERT KO rats after chronic imipramine treatment.

4. Discussion

In the current study, female rats demonstrated increased signs of mania-like behavior such as increased asocial behavior, altered problem-solving capability and hyperactivity under chronic imipramine treatment. This phenotype was more pronounced in SERT KO than in WT rats (Supplementary Table 2). Imipramine treatment also decreased anxiety and induced rigidity regardless of genotype. We further found that chronic imipramine treatment increased the expression of the BDNF-TrkB-Akt pathway in the infralimbic, but not in the prelimbic, cortex of SERT KO rats. Thus, our study indicates that female SERT KO rats are more sensitive to imipramine treatment, suggesting that female BD patients with the 5-HTTLPR s-allele might be at a higher risk of a switch from depression to mania under imipramine treatment. This phenotype correlates with, at least partly, the activating signaling of the BDNF-TrkB-Ark pathway in the infralimbic cortex.

The cumulative evidence shows that SERT KO rats exhibit an altered ability to cope with stress and display increased innate anxiety and depression-like behaviors (Holmes et al., 2002; Adamec et al., 2006; Holmes et al., 2003; Jansen et al., 2010; Kalueff et al., 2010). These features are reflected in the behavioral tests such as anxiety-like avoidance of open arms in the elevated plus maze test (Olivier et al., 2008; Kalueff et al., 2010) and an increased aversion to the center in the open field test (Olivier et al., 2008). Such increased anxiety may contribute to explain the reduced social behavior at some level (Homberg et al., 2007a; Chan et al., 2011; Kiser et al., 2012). Our study shows that, at baseline, SERT KO rats demonstrate a preference for closed arm (elevated plus maze test, $P < 0.05$, *T*-Test) and avoidance of the central area (open field test, second 30 min, $P < 0.01$, T-Test) compared to WT rats. These observations are consistent with previous reports (Olivier et al., 2008; Kalueff et al., 2010) and validate our SERT KO model. SERT KO rats can also display increased flexibility and sensitivity to environmental stimuli with positive or negative responses (Carola and Gross, 2012). For example, these rats manifest improved cognitive flexibility in the reversal learning task (Nonkes et al., 2013; Brigman et al., 2010) and sensitivity to stress (Schipper et al., 2019) and environmental enrichment (Nonkes et al., 2014; Sbrini et al., 2022). This feature is seen in the puzzle box test and home-cage test. Interestingly, in the open field test, both SERT KO and WT rats spent less time in the center of the arena after the imipramine treatment, suggesting the treatment does not release the anxiety-like behavior as expected. However, this result conflicts with the pharmacological actions of imipramine in the elevated plus maze test. A previous study showed that decreased time in the center could also result from thigmotaxis or cognitive rigidity (Reinwald et al., 2022). This rigidity can point to stereotyped behavior: moving along the walls due to an inability to flexibly change behavior and thereby avoiding moving to the center. Therefore, we cannot rule out that SERT KO and WT rats exhibited rigidity in the open field test due to the potential side effect of imipramine.

In rodents, mania-like behaviors in BD are extremely difficult to mimic and are generally modeled by cognitive deficits, perturbations in circadian rhythms, and hyperactivity (Li et al., 2022; Pappas et al., 2017; Jaubert et al., 2007; Szumlinski et al., 2005). We found that chronic imipramine treatment induced executive dysfunction, abnormal circadian rhythms and increased locomotor activity specifically in SERT KO compared to WT rats. The longer latency in puzzle box test is reminiscent of deficiency of close-up problem solving, while it is also strongly affected by motivation, locomotor activity, and anxiety levels (Ben Abdallah et al., 2011). Because rodents prefer darkness and avoid lightness, the increased locomotor activity during light phase may represent dysregulation and hyperactive behavior in the sleeping phase. It could also mirror risk-taking and fearless behaviors. This is critical as such behaviors are hallmarks of BD mania. Of note, the interactions between symptoms, which could be understood as a network, may rise the mental disorders eventually (Borsboom, 2017). The behavioral manifestations of the SERT KO rats could result from the interaction between anxiety, depression or sensitivity, and finally be facilitated by imipramine through some specific neural pathways (see below). Therefore, our study might recapitulate how chronic imipramine treatment induces mania switch in female BD patients carrying the 5-HTTLPR s-allele in the clinic.

From an anatomical point of view, the medial prefrontal cortex can

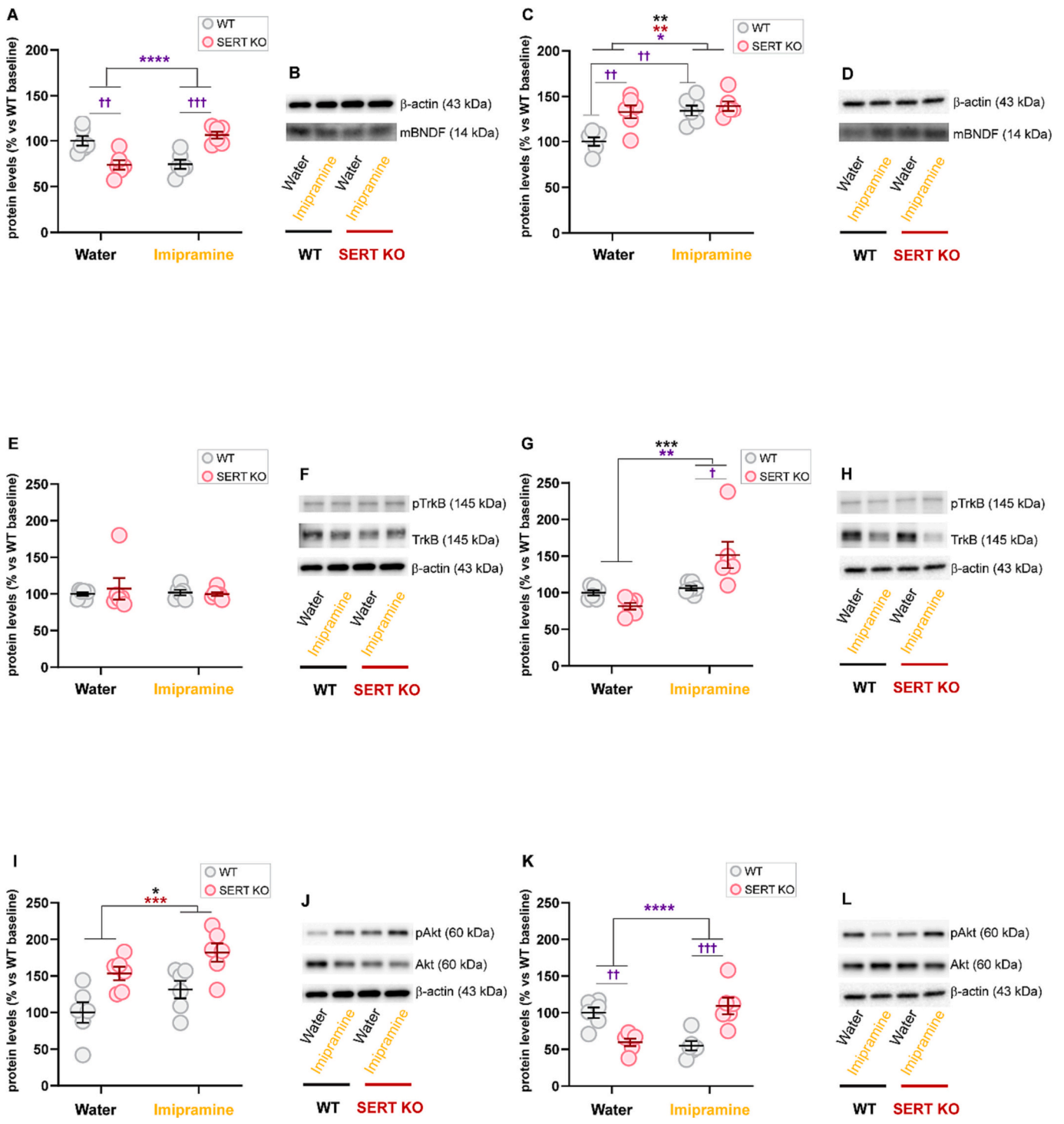

Fig. 7. Interaction between SERT KO and imipramine treatment on the BDNF system in the prelimbic and infralimbic cortices. (A) mBDNF protein expression levels, (E) the ratio of pTrkB/TrkB and (I) the ratio of pAkt/Akt in the whole homogenate of prelimbic cortex in WT and SERT KO rats treated with water or imipramine. (C) mBDNF protein expression levels, (G) the ratio of pTrkB/TrkB and (K) the ratio of pAkt/Akt in the whole homogenate of infralimbic cortex in WT and SERT KO rats treated with water or imipramine. Representative immunoblots related to the expression levels of mBDNF (B), pTrkB and TrkB (F), pAkt and Akt (J) measured in the prelimbic cortex of WT and SERT KO rats. Representative immunoblots related to the expression levels of mBDNF (D), pTrkB and TrkB (H), pAkt and Akt (L) measured in the infralimbic cortex of WT and SERT KO rats. Each circle represents an individual rat. The horizontal bar and error bars represent mean ± S.E.M. *$P <$ 0.05, **P < 0.01, and ***P < 0.001 indicate a significant effect of treatment; **P < 0.01 and ***P < 0.001 indicate a significant effect of genotype; *$P <$ 0.05, **P < 0.01, and ****P < 0.0001 indicate a significant effect of the interaction of treatment × genotype (Mixed model, $n = 6$ per group). †P < 0.05, ††$P <$ 0.01, †††P < 0.00 indicate a significant difference between groups under the same condition or the same group under different conditions (Tukey's multiple comparison post hoc test). WT = wild-type rats; SERT KO = serotonin transporter knock-out rats.

be divided into prelimbic and infralimbic cortices (Heidbreder and Groenewegen, 2003; Ongür and Price, 2000). Although these two brain regions are located extremely close together in the brain, they exhibit different roles. Nonetheless, they are both involved in regulating fear expression. It has been extensively shown that BDNF in the prefrontal cortex is required for fear memory processing (Cowansage et al., 2010; Bekinschtein et al., 2008) and consolidation (Bambah-Mukku et al., 2014; Kim et al., 2012), acting via activation of TrkB. BDNF in the prelimbic cortex appears to be necessary for the consolidation of fear conditioning (Choi et al., 2010; Choi et al., 2012; Ye et al., 2017), while the infralimbic cortex undergoes plasticity for inhibition of fear response (Kim et al., 2011; Sotres-Bayon and Quirk, 2010). In other words, fear extinction is enhanced by BDNF expression in the infralimbic cortex (Peters et al., 2010). Recent studies elucidate that the chronic administration of imipramine increases the BDNF-TrkB interaction in the rat cingulate cortex (Faron-Górecka et al., 2022; Casarotto et al., 2021). Our study demonstrates clear differences between prelimbic and infralimbic cortices in the modulation of the BDNF downstream pathway. We found that imipramine administration led to a significant activation of TrkB and Akt in the infralimbic of SERT KO only, while no effects were observed in the prelimbic cortex of both genotypes. The possibility exists that imipramine could favor the switch to mania-like behavior in female SERT KO rats by activating the BDNF downstream pathway in the infralimbic cortex. Our hypothesis is that the imipramine-induced increase of signaling in SERT KO rats might favor the extinction of fear and, thus, the engagement in risky behaviors. Despite the behavioral tests we employed did not specifically address fear extinction, our study might partly explain the neural pathway of risk-taking and impulsive behaviors in the mania episode of female patients with BD.

An important feature of our study is that we used both male and female rats. The female rats were found to be more sensitive to the effects of imipramine than the male rats. In general, female subjects are underrepresented in animal research across disciplines (Beery and Zucker, 2011). Only 9 % of the studies in the field of neuroscience use female animals (Beery, 2018), despite it was reported that, in rats, there are no sex differences in overall variability (Becker et al., 2016). Lack of basic research on female animals may result in less efficient treatment outcomes for female patients (Klein et al., 2015). Because female BD patients more often undergo the switch from depression to mania under imipramine treatment, we used female rats to understand the underlying neural mechanisms. Our female SERT KO rats mirrored female BD patients carrying the 5-HTTLPR s-allele suffering from the switch to mania under imipramine treatment. Accordingly, our data suggest that these patients may be at increased risk of a switch to mania under the imipramine treatment via overexpression of the BDNF-TrkB-Akt pathway in the infralimbic cortex.

This study has strengths as well as limitations. A strength of this study is that we applied a longitudinal study design and tested the switch from depression-like behavior to mania-like behavior within the same animals. A limitation of this is that repeated testing could potentially affect the results of the behavioral tests. For example, the elevated plus maze test is considered sensitive to repeated tests (Carobrez and Bertoglio, 2005; File et al., 1990). Yet pioneering results of some studies revealed a stable outcome in repeated tests (Pellow et al., 1985; Lister, 1987). A recent study showed that measures of general performance such as total distance and open arm avoidance are stable across repeated elevated plus maze tests (Schrader et al., 2018). Moreover, we observed that imipramine had selective behavioral effects in the elevated plus maze test. Hence, we carefully assume that the observed effects of imipramine treatment are not reflecting repeated testing effects. Another limitation of our study is that we measured the imipramine induced changes in behavior and in the BDNF system in separate animals. The reason was to avoid the influences of behavioral testing itself on BDNF levels. A disadvantage of this choice is that we cannot directly associate the behaviors with molecular findings. Investigation of the potential causal link between the two awaits future research.

In conclusion, to the best of our knowledge, this is the first study to examine the neural pathway involved in the switch from anxiety- and depression-like to mania-like behavior using the female SERT KO rat model. We found that the BDNF-TrkB-Akt pathway in the infralimbic, but not prelimbic, cortex changed in response to imipramine, specifically in the female SERT KO rats. While causal evidence is still lacking, this may suggest that this pathway in the infralimbic cortex contributes, at least in part, to the switch to mania-like behavior. Given the translational nature of the SERT KO rat model (Schipper et al., 2019), these findings may have implications for female BD patients carrying the 5-HTTLPR s-allele. A clear understanding of the imipramine induced switch to mania will aid in the improvement of the treatment of BD.

CRediT authorship contribution statement

Mina Sadighi: Writing – review & editing, Data curation, Conceptualization. **Lingling Mai:** Writing – review & editing, Writing – original draft, Formal analysis. **Yifan Xu:** Writing – review & editing. **Morgane Boillot:** Writing – review & editing, Data curation. **Giorgia Targa:** Data curation. **Francesca Mottarlini:** Data curation. **Paolo Brambilla:** Funding acquisition. **Peter Gass:** Writing – review & editing. **Lucia Caffino:** Data curation. **Fabio Fumagalli:** Writing – review & editing, Funding acquisition. **Judith R. Homberg:** Writing – review & editing, Writing – original draft, Supervision, Conceptualization.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jad.2024.01.186.

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