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Full-length Article

Perinatal serotonergic manipulation shapes anhedonic and cognitive behaviors in a sex- and age-dependent manner: Identification of related biological functions at central and peripheral level

Maria Teresa Gallo, Paola Brivio *, Beatrice Dolci, Fabio Fumagalli, Francesca Calabrese

Department of Pharmacological and Biomolecular Sciences "Rodolfo Paoletti", Università Degli Studi di Milano, Milan, Italy

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ABSTRACT

Poor knowledge about psychiatric disorders often results in similar diagnoses for patients with different symptoms, thus limiting the effectiveness of the available medications. As suggested by several lines of evidence, to improve these shortcomings, it is essential to identify biomarkers associated with specific symptoms and to stratify patients into more homogeneous populations taking a further step toward personalized medicine.

Here, we aimed to associate specific behavioral phenotypes with specific molecular alterations by employing an animal model based on the pharmacological manipulation of the serotonergic system, which mimics a condition of vulnerability to develop psychiatric disorders. In particular, we treated female and male rats with fluoxetine (FLX 15 mg/kg dissolved in drinking water) during prenatal or early postnatal life, and we evaluated different pathological-like phenotypes (cognitive deficit, anhedonia, and anxiety) by exposing the rats to a battery of behavioral tests during adolescence and adulthood. In addition, we carried out molecular analyses on specific brain areas and in the blood.

Our results showed that perinatal FLX administration determined age- and sex-dependent effects, with males being more sensitive to prenatal manipulation and manifesting anhedonic-like behavior and females to early postnatal exposure, exhibiting cognitive deficits and a less anxious phenotype. Furthermore, we identified, peripherally and centrally, biological functions altered by perinatal serotonin modulation regardless of the timing of exposure and sex, and other pathways specific for the pathological-like phenotypes.

The results presented here provide new insights into potential biomarkers associated with specific behavioral phenotypes that may be useful for broadening knowledge about psychiatric conditions.

1. Introduction

Although psychiatric disorders commonly lead to severe and long-term disabilities and are major contributors to the global socioeconomic burden (James et al., 2018), they are still poorly understood due to their complexity and heterogeneity. Indeed, an incomplete knowledge of these pathological conditions results in few treatment options and similar diagnoses among patients who, however, show very different symptoms (Howes et al., 2022).

To facilitate diagnosis and find more effective treatments, it is indeed helpful to stratify patients into more homogeneous populations to identify biomarkers associated with specific symptoms to take a further step toward personalized medicine.

Due to the limitations of clinical research, most studies in this field use rodent models that mimic psychiatric conditions. Because of the consolidated importance of the gene X environment interaction in the onset of psychiatric pathologies, many of these studies employ transgenic models to investigate the functional role of a particular gene. Specifically, given the crucial role of serotonin (5-HT) in the pathophysiology of psychiatric disorders (Pourhamzeh et al., 2022) and the evidence of functional polymorphism in the promoter region of the serotonin transporter (5-HTT or SERT) (Caspi et al., 2010, 2003), over the years we have focused on animal models with genetic alterations related to essential determinants of the serotonergic system. We conducted studies in 5-HTT knockout (5-HTT$^{-/-}$) rats (Brivio et al., 2022, 2019; Calabrese et al., 2015; Homberg and Lesch, 2011; Sbrini et al., 2020a),

* Corresponding author at: Department of Pharmacological and Biomolecular Sciences "Rodolfo Paoletti", Università Degli Studi di Milano, Via Balzaretti 9, 20133 Milan, Italy.
E-mail address: paola.brivio@unimi.it (P. Brivio).

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but also in animals lacking the genes coding for tryptophan hydroxylase (TPH), the rate-limiting enzyme in the synthesis of 5-HT respectively in periphery, the TPH1$^{-/-}$ rats (Sbrini et al., 2022), and in the central nervous system, the TPH2$^{-/-}$ rats (Brivio et al., 2018; Meng et al., 2022; Sbrini et al., 2020b).

Nevertheless, although these models have significantly advanced the knowledge about the mechanisms underlying mental disorders, genetic approaches hold some limitations given the versatility that characterizes the serotonergic system, its development, and its functions over time. Furthermore, genetic manipulations often lead to life-long alterations that can trigger compensatory changes or adaptations in serotonergic and other neurotransmitter systems and complicate the interpretation of the observed findings (Hainer et al., 2015). In addition, these approaches do not allow identifying potential periods of vulnerability critical to understanding the nature of the pathological condition and do not offer the opportunity to study specific time-related interventions.

Another crucial point in the field of mental health research is the sex-dependent incidence of psychiatric disorders. Indeed, the risk of developing mood disorders, such as depression and anxiety disorders, is at least two times higher in women than in men (Altemus et al., 2014). Conversely, neurodevelopmental disorders, such as intellectual disability, autism spectrum disorders, and attention-deficit hyperactivity disorder, occur two to four times more frequently in men than women (May et al., 2019).

On these bases, we decided to manipulate the serotonergic system in the prenatal and early postnatal life using a pharmacological approach to investigate the long-lasting effects of serotonin modulation on the development of depressive-anxious-like behavior in adolescent and adult male and female rats. Specifically, we administered the selective serotonin reuptake inhibitor (SSRI) fluoxetine (FLX) during pregnancy and during the breastfeeding period, life phases in which 5-HT plays a crucial role in the development. Indeed, it acts as a neuromodulator and trophic factor, influencing processes such as neurogenesis, synaptic plasticity, and cell division (Azmitia, 2001). In line with these considerations, perinatal exposure to SSRIs has been shown to alter hippocampal plasticity (Karpova et al., 2009), induce changes in the formation of perineuronal nets (Mukhopadhyay et al., 2021), and disrupt hippocampal neurogenesis in a sex-related manner (Gemmel et al., 2016). Accordingly, early-life exposure to SSRIs can impact brain development and cause behavioral consequences later in life (Kepser and Homberg, 2015).

Furthermore, based on the already mentioned heterogeneity of symptoms characterizing these pathological situations, we decided to evaluate the influence of early-in-life FLX exposure by studying the response to three different tests which investigate diverse domains that concern, albeit in different ways, the depressive phenotype. Indeed, we performed the sucrose preference test (SPT) to assess the hedonic-like condition, the novel object recognition (NOR) test to study cognitive abilities, and the elevated plus maze (EPM) test to evaluate anxious behaviors.

We conducted molecular studies based on the behavioral outcome obtained in adult male and female rats to identify potential molecular mechanisms underlying the observed pathological phenotypes. To this aim, we analyzed the expression of genes involved in different processes: synaptic plasticity, hypothalamic–pituitary–adrenal axis, immediate early genes, early responsive genes, autophagy-related genes, mitochondrial activity-related genes, and proinflammatory cytokines. We focused on brain regions known to be involved in psychiatric conditions, such as the prefrontal cortex (PFC) and the dorsal and ventral subregions of the hippocampus (respectively dHip and vHip) (Fanselow and Dong, 2010; Hiser and Koenigs, 2018; Price and Duman, 2020). Lastly, by searching for peripheral biomarkers, we conducted the same molecular analysis in the blood.

Our results show that perinatal administration of FLX leads to different pathological phenotypes and molecular alterations in a sex- and age-dependent manner that also depend on the perinatal period of manipulation.

2. Material and methods

2.1. Animals

Adult female and male Wistar rats (Charles River, Germany) were brought into the laboratory one month before the start of the experiment. Then, each female was coupled with a male and after the mating, the males returned to their original cages, while the females were singly housed with the nest material.

At birth date, the offspring remained with their mother until the weaning at postnatal day (PND) 21, when males and females were separated and housed in groups of 2–4 animals per cage. Food and water were freely available on a 12-h light/dark constant temperature (22 ± 2 °C) and humidity (50 ± 5 %). The number of animals used in this experiment and the size of each experimental group is outlined in Fig. 1B.

All procedures used in this study were conducted in accordance with the authorization n. 472/2021-PR approved by the Italian Health Ministry in line with the Italian legislation in animal experimentation (DL 26/2014) and conformed to the European Communities Council Directive of September 2010 (2010/63/EU). All efforts were made to minimize animal suffering, to reduce the number of animals used, and the animal study complies with the ARRIVE guidelines.

2.2. Experimental paradigm and drug administration

After the mating, dams were randomly divided into experimental groups. As shown in Fig. 1A, we administered the SSRI FLX during pregnancy, from gestational day (GD) 0 to PND0 or breastfeeding, from PND0 to PND21. In particular, 7 dams were exposed to FLX during pregnancy and vehicle during breastfeeding (group prenatal-FLX), 6 received the drug only throughout breastfeeding (postnatal-FLX), and 6 others were assigned to the control group (vehicle) taking vehicle during both periods considered (for details about litters composition see Supplementary Tables 1 and 2).

The drug was dissolved directly in the drinking water, and the dams assumed 15 mg/kg/day. The solution was prepared in excess according to the amount of water animals drink daily. To obtain the correct dosage, dams were weighed, and the volume of water consumed over the previous two days was measured. These measurements were used to calculate the amount of FLX to be dissolved in water to approximate proper intake. This protocol is intended to correct for changes in body weight and water consumption inherent to pregnancy.

At the end of the pharmacological manipulation, all the animals were left undisturbed in their home cages until the behavioral tests. Part of the offspring was exposed to SPT, NOR, and EPM during adolescence (PND35) and the other in adulthood (PND70).

2.3. Behavioral tests

The behavioral test battery was performed on the male and female offspring during adolescence (PND35) and in adulthood (PND70) in the following order: SPT, NOR, and EPM (Fig. 1A). The specific order of the tests was chosen to go from least to most invasive to minimize the risk that the previous testing procedures influenced the responses to the following ones. Behavioral testing was done blindly by an experimenter unaware of the experimental group of animals.

Sucrose preference test

As previously described (Caffino et al., 2020) the animals were habituated to drink water from two bottles in their home cage for three days before starting the test. After that, all animals were subjected to 15 h of food and water deprivation and then housed individually. During the test, they were presented with one bottle containing 1% sucrose solution and another one with water for 1 h. The position of the sucrose

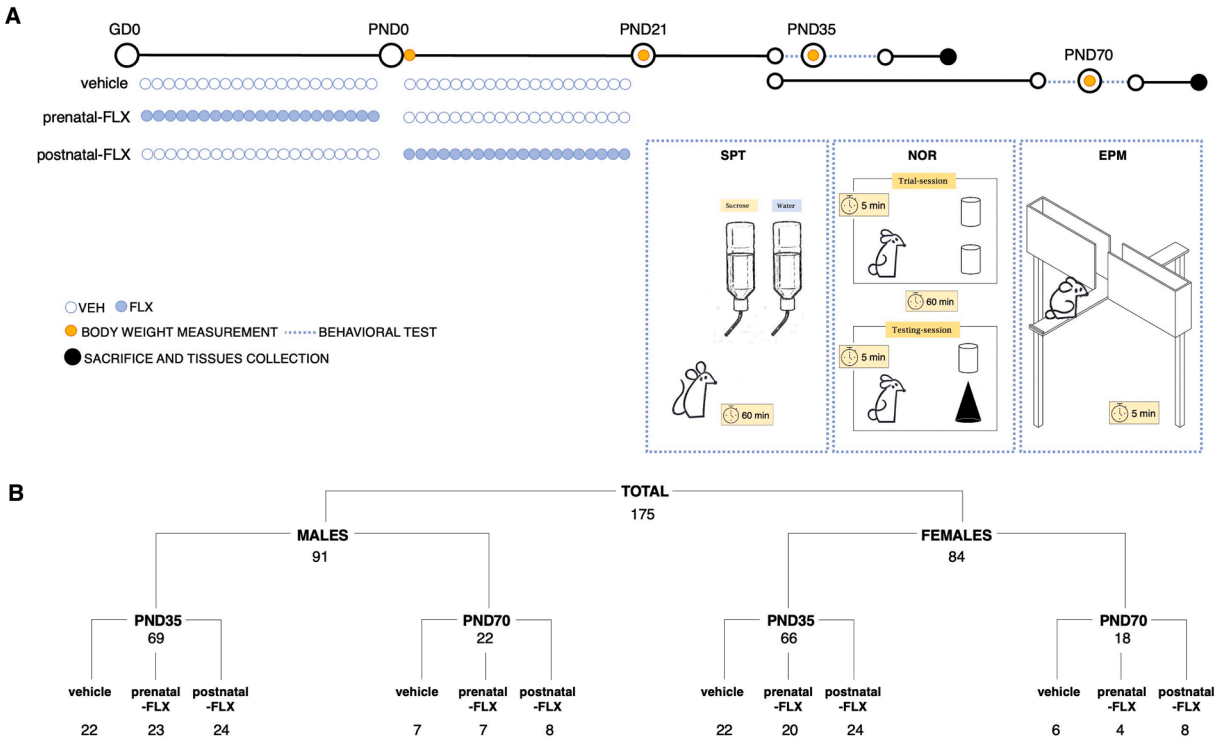

Fig. 1. Schematic representation of the experimental paradigm. Panel A: Male and female Wistar rats were exposed to fluoxetine (FLX) or vehicle (VEH) during gestation or breastfeeding. We registered the body weight at PND1, 21, 35 and 70. The animals were subjected to the sucrose preference test (SPT), the novel object recognition (NOR) test and the elevated plus maze (EPM) test during adolescence or at adulthood and sacrificed the week after. Panel B summarizes the number of rats used in the experiment divided into the different experimental groups.

solution was determined as the less favorite by the animals during the habituation period to prevent spatial bias.

The sucrose preference was calculated as [(ml of sucrose solution drunk) / (total ml of fluids drunk)] × 100.

Novel object recognition test

The animals were tested in a non-transparent squared open field (50x50x40 cm) (Fig. 3C,4C), and the test protocol (see Brivio et al., 2020) consisted of three phases: five minutes of training (two identical plastic bottles), one hour of inter-trial interval in the home cage, and five minutes of test (one plastic bottle and one tin can). Automatic assessment (ANY-maze software, Ireland) was averaged with the manual scoring of the time spent exploring the familiar object and the novel one. The NOR index was calculated by applying the following formula: [(time exploring the novel object/time exploring both the novel and familiar objects)] X 100.

Elevated plus maze test

Animals were tested in an elevated plus maze (raised 60 cm above the ground), which consists of two open arms (OA) and two closed arms (CA), enclosed by 40 cm high walls, 50 cm long, and 10 cm wide (Fig. 3D,4D). As described in (Bosch et al., 2022), animals were placed in the center of the maze and they were allowed to explore the EPM for 5 min, during which we registered the time spent, the distance moved in each area (OA, CA and center) and the number of entries in the OA, both manually and using ANY-maze software.

2.4. Tissues collection

One week after the behavioral tests, animals were decapitated. Trunk blood was collected in RNA protect Animal Blood Tubes (Qiagen, Italy) and stored at −20 °C following the manufacturer's instructions. The prefrontal cortex (PFC), dorsal hippocampus (dHip) and ventral hippocampus (vHip) were dissected from the whole brain, frozen on dry ice and stored at −80 °C for later analyses. Precisely, the PFC corresponds to plates 6–13, dHip to 43–72 and vHip to 73–84 according to the atlas of Paxinos and Watson (Paxinos and Watson, 2007).

2.5. RNA preparation and gene expression analysis by quantitative real time PCR

Total RNA was isolated from the frozen brain tissues by single step of guanidinium isothiocynate/phenol extraction using PureZol RNA isolation reagent (Bio-Rad Laboratories, Segrate, Italy) and quantified by spectrophotometric analysis as previously described (Brivio et al., 2021b). To avoid DNA contamination, samples were treated with DNase (ThermoFisher scientific, Italy).

We used the RNeasy Protect Animal Blood Kit (Qiagen, Italy) following the manufacturer's protocol to isolate the RNA from the blood.

Real-time polymerase chain reaction (q-PCR) was performed to assess *Arc, c-Fos, Zif268, Nr4a1, Dusp1, Fkbp5, Sgk1, Gilz, Gadd45β, Grα, Grβ,* Total *Bdnf, Bdnf* long 3′ UTR, *Bdnf* isoform IV, *Bdnf* isoform VI, *Ulk1, Becn1, Map1lc3, Ctsb, Cox1, Cox3, Pgc1α, Gpx1, Il1β, Tnfα* mRNA levels. Primers sequences used were purchased from Eurofins MWG-Operon (Supplementary Table 3) and Life Technologies (Supplementary Table 4).

RNA was analyzed by TaqMan qRT-PCR instrument (CFX384 real time system, Bio-Rad Laboratories, Italy) using the iScriptTM one-step RT-PCR kit for probes (Bio-Rad Laboratories, Segrate, Italy) (see (Brivio et al., 2023) for details). Samples were run in 384 well formats in triplicate as multiplexed reactions with the normalizing internal control *36b4*.

2.6. Functional enrichment analysis

The target genes' expression levels were analyzed by using the WEB-based Gene SeT AnaLysis Toolkit (WebGestalt) (Liao et al., 2019), a powerful online tool for functional enrichment analyses. We performed the Gene Ontology (GO) enrichment analysis that includes biological process, cellular component and molecular function, and the pathway

analysis considering three different databases: KEGG, WikiPathways and Reactome. Notably, for these investigations, we used the Gene Set Enrichment Analysis (GSEA) method.

2.7. Statistical analysis

All the results were analyzed using GraphPad Prism software version 8.0 (GraphPad Software Inc., CA, United States).

The Unpaired *t*-test was used to analyze the birth rate. In contrast, the body weight was analyzed using the two-way analysis of variance (ANOVA) with repeated measures, with treatment and age as independent factors, followed by the Tukey multiple comparison test.

The behavioral and molecular data were analyzed with one-way ANOVA, with the treatment as variable, followed by the Tukey multiple comparison test.

Significance for all the tests was assumed for $p < 0.05$. Data are presented as Gardner-Altman plots (Fig. 2A) or as mean ± standard error (SEM) (Fig. 2B-6).

3. Results

3.1. Perinatal FLX affects birth rate and body weight

We first evaluated the influence of the alteration of the serotonergic system during prenatal life on births. As represented in Fig. 2A, we observed that the mean number of pups born from FLX group dams was significantly lower than the control one (-3.1, $p < 0.05$. Unpaired *t*-test). Furthermore, two-way ANOVA with repeated measures revealed a significant effect of FLX on the body weight in both males ($F_{2,90} = 5753$, $p < 0.001$) and females ($F_{2,83} = 4671$, $p < 0.001$). In particular, Tukey's multiple comparison test showed that the lower body weight was due to the postnatal more than the gestational administration (Fig. 2B and Supplementary Table 5). Notably, this effect was present in postnatal-FLX rats at all ages considered.

Interestingly, in rats exposed to FLX during gestation, the reduction of the body weight was explicitly found at PND1 (data not shown). At the same time, as illustrated above, the consequences of postnatal FLX persisted until adulthood and cannot be ascribed to a reduced food intake (Supplementary Table 6).

These data suggest that the prenatal alteration of the serotonergic system results in a reduced birth rate and lower-weight offspring. However, pups exposed to prenatal FLX regained weight at weaning. Conversely, early postnatal exposure to FLX is associated with lower body weight that persists during the lifetime.

3.2. Prenatal FLX induces anhedonic-like phenotype selectively in adult male rats

As shown in Fig. 3 (panels A and B), one-way ANOVA did not reveal any difference due to the perinatal pharmacological administration during adolescence in either males ($F_{2,40} = 3.051$, $p > 0.05$) or females ($F_{2,40} = 0.290$, $p > 0.05$) in the SPT, while in adults a significant effect was observed in male ($F_{2,20} = 6.19$, $p < 0.01$) (Fig. 4A), but not in female ($F_{2,15} = 0.8637$, $p > 0.05$), rats (Fig. 4B). In particular prenatal FLX led to reduced sucrose preference in males compared to the vehicle group (-54.1%, $p < 0.01$. Tukey's multiple comparison test).

This result indicates that the prenatal alteration of the serotonergic system induces an anhedonic-like phenotype in a sex- and age-dependent manner.

3.3. Postnatal FLX is associated with cognitive deficits specifically in adult female rats

In adolescent rats one-way ANOVA did not show differences in the performance in the NOR (males: $F_{2,38} = 0.096$, $p > 0.05$; females: $F_{2,41} = 0.4634$, $p > 0.05$) (Fig. 3E-H), whereas at adulthood we observed significant effect of the pharmacological manipulation selectively in females ($F_{2,17} = 4.42$, $p < 0.05$) (Fig. 4G, H), but not in males ($F_{2,21} = 1.382$, $p > 0.05$) (Fig. 4E, F). Indeed, the multiple comparisons revealed a reduction in the NOR index % only in adult female rats exposed to FLX during breastfeeding compared to the vehicle group (–32.9%, $p < 0.05$. Tukey's multiple comparison test). This effect cannot be ascribed to changes in locomotor activity (Supplementary Table 7).

These results suggest that the perinatal dysregulation of 5-HT is associated with cognitive impairments depending on the sex and the period of serotonergic alteration.

3.4. Female rats exposed to perinatal FLX show a higher impulsivity

As shown in Fig. 3I-P, one-way ANOVA underlined significant effects of FLX in the EPM test during adolescence specifically in females (open arms: $F_{2,65} = 6.930$, $p < 0.01$; closed arms: $F_{2,65} = 2.972$, $p = 0.0584$). In particular, Tukey's multiple comparison test (Supplementary Table 8) highlighted a less anxious phenotype in the females belonging to the prenatal-FLX group (open arms: +33.5 s, $p < 0.01$; closed arms: −38.5 s, $p < 0.05$) compared to the control group and, interestingly, this effect persisted into adulthood (open arms: +63 s, $p < 0.05$; closed arms: −73.8 s, $p < 0.01$), when it also appeared in the females of the postnatal-FLX group (open arms: +45.2 s, $p < 0.01$; closed arms: −52.1 s, $p < 0.05$) (Fig. 4M-P). Accordingly, in adulthood, one-way ANOVA showed a significant effect of the pharmacological manipulation on time spent in the open and closed arms (open arms: $F_{2,17} = 5.775$, $p < 0.05$; closed arms: $F_{2,17} = 7.732$, $p < 0.01$) specifically in female (Fig. 4M-P) but not in male animals (open arms: $F_{2,21} = 2.802$, $p > 0.05$; closed arms: $F_{2,21} = 0.6574$, $p > 0.05$) (Fig. 4I-L).

These results suggest that the perinatal alteration of the 5-HT system selectively drives the female rats to explore the EPM apparatus more than the control group. Additionally, consistent with this phenotype, we

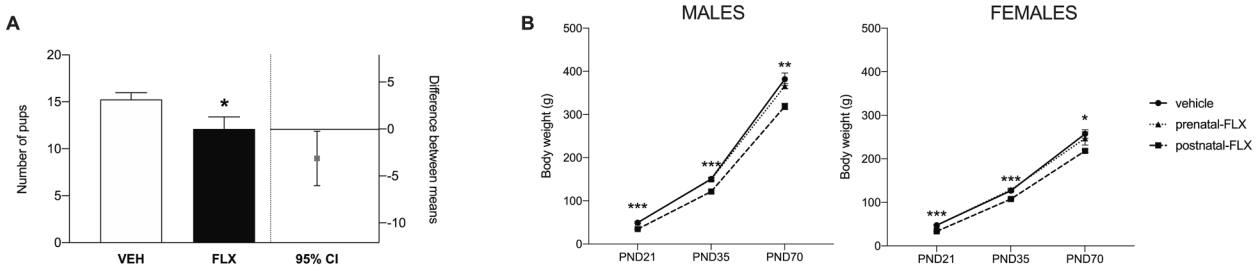

Fig. 2. Birth rate and body weight of rats exposed to FLX during perinatal periods. Panel A shows the number of pups born from dams exposed to FLX or vehicle (VEH) during gestation. The results are presented as Gardner-Altman plots. The red square indicates the difference between the two means, and the error bars show the 95% confidence interval of that difference. The data are expressed as mean ± SEM of independent determinations. *$p < 0.05$ vs VEH (Unpaired *t*-test). Panel B shows the body weight of male (left) and female (right) rats of prenatal-FLX, postnatal-FLX and VEH groups at PND 21, 35 and 70. The data are expressed as mean ± SEM of independent measures. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs vehicle at the same age (two-way ANOVA with repeated measures followed by Tukey's multiple comparison test).

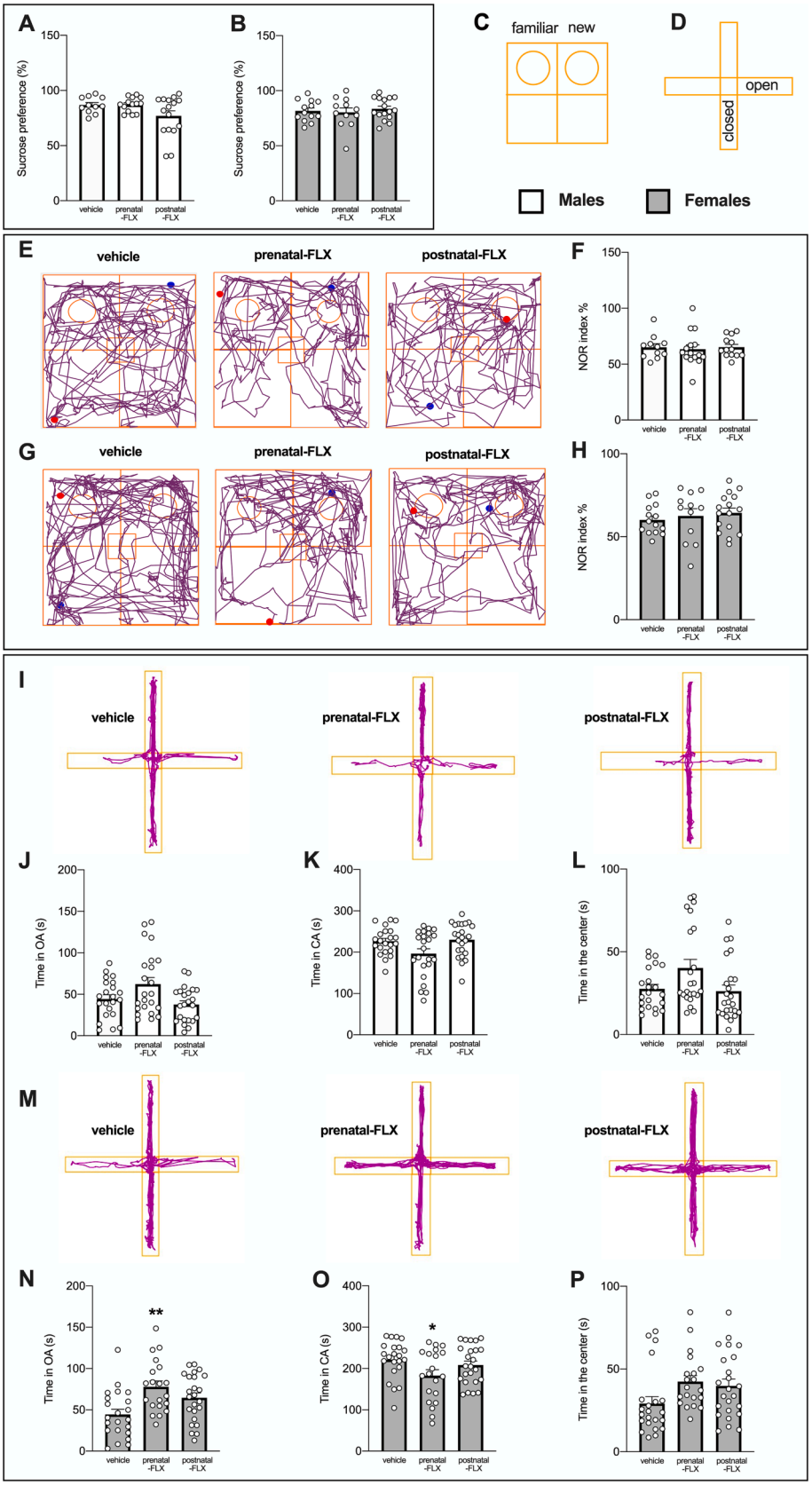

Fig. 3. Behavioral tests in adolescent rats exposed to perinatal FLX. Panel A (males) and B (females): Sucrose preference %. Panel C: schematic representation of the novel object recognition (NOR) test arena. Panel D: schematic representation of elevated plus maze (EPM) platform. Panel E (males) and G (females): Track plots showing the animal's path during the test phases of the NOR. The blue and red dots are respectively the starting and ending points. Panel F (males) and H (females): NOR index %. Panel I (males) and M (females): Track plots showing the animal's path during the EPM test. Panels J, K, L (males) and N, O, P (females): time spent respectively in the open arms, in the closed arms, and in the center of the EPM. Data are expressed as mean ± SEM of independent measures. $^{*}p < 0.05$, $^{**}p < 0.01$ vs vehicle (one-way ANOVA with Tukey multiple comparison test).

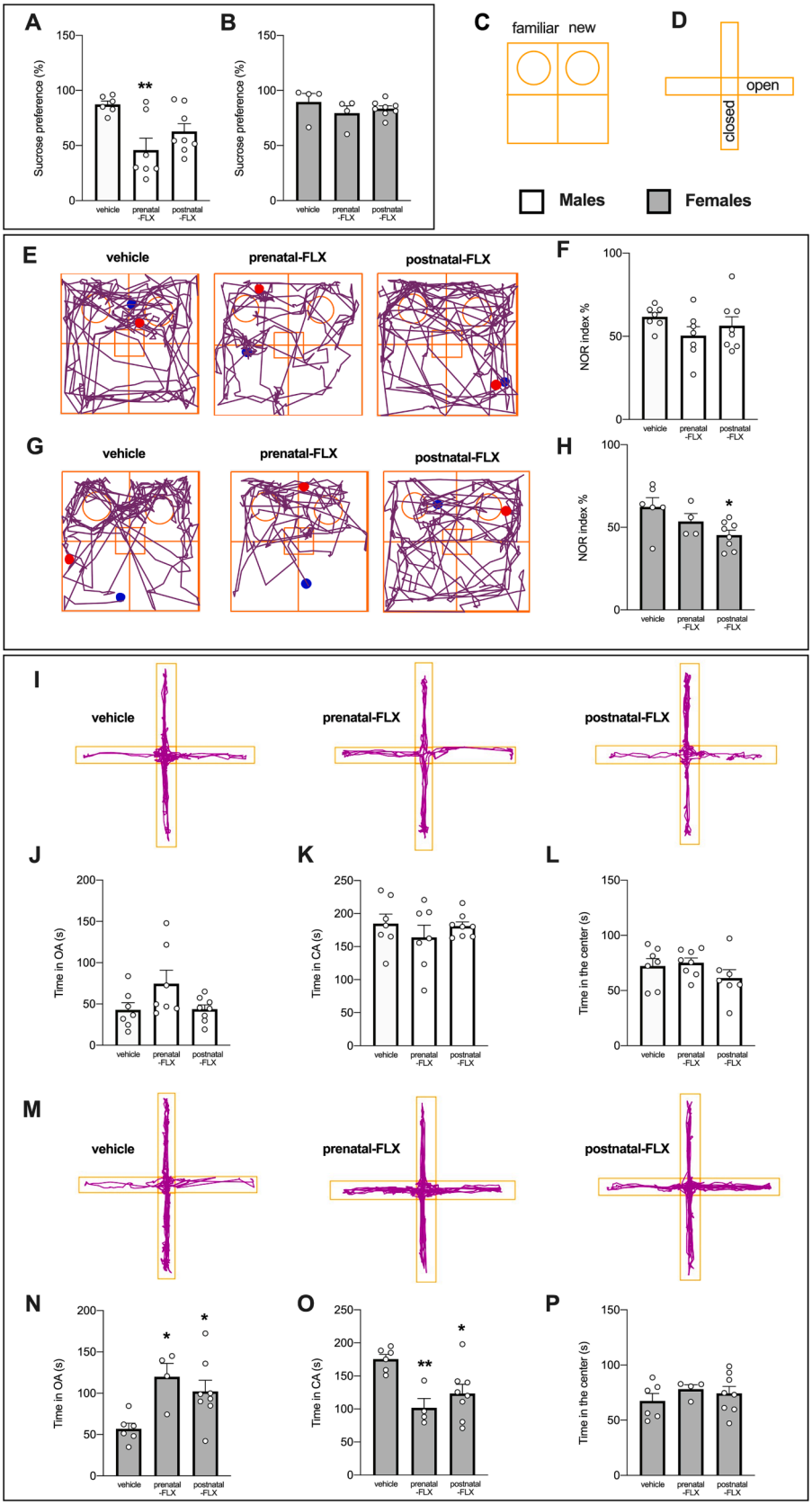

Fig. 4. Behavioral tests in adult rats exposed to perinatal FLX. Panel A (males) and B (females): Sucrose preference %. Panel C: schematic representation of the novel object recognition (NOR) test arena. Panel D: schematic representation of elevated plus maze (EPM) platform. Panel E (males) and G (females): Track plots showing the animal's path during the test phases of the NOR. The blue and red dots are respectively the starting and ending points. Panel F (males) and H (females): NOR index %. Panel I (males) and M (females): Track plots showing the animal's path during the EPM test. Panels J, K, L (males) and N, O, P (females): time spent respectively in the open arms, in the closed arms, and in the center of the EPM. Data are expressed as mean ± SEM of independent measures. *$p < 0.05$, **$p < 0.01$ vs vehicle (one-way ANOVA with Tukey multiple comparison test).

observed that the treated females entered the open arms more times than the control group at both PND35 and 70 (data not shown).

3.5. Gene expression changes in the blood of rats exposed to FLX

To identify potential peripheral markers associated with the perinatal modulation of the serotonergic system, we measured the expression of different classes of genes, such as markers of neuronal plasticity, autophagy, mitochondrial activity and proinflammatory cytokines in the blood of adult rats.

Overall, we observed that most of the targets analyzed were increased in the blood of treated animals compared to the control groups (Fig. 5A, Supplementary Tables 9 and 10). The results were processed through the GO enrichment analysis considering biological process, cellular component and molecular function categories (Fig. 5D). Interestingly, we found that 11 categories were modulated regardless of the period of exposure to FLX and the sex, while others were specific for each experimental group (12 in males prenatal-FLX, 9 in males postnatal-FLX, 4 in females prenatal-FLX and 6 in females postnatal-FLX). Moreover, we found 2 common GO categories among males and 2 common among females (Fig. 5B and Supplementary Table 11).

Focusing on the GO categories found explicitly in the groups showing the pathological phenotype, we found that anhedonic males (prenatal-FLX) showed modulation of the so-called "mitochondrion organization", "muscle tissue development", "neuron death", "generation of precursor metabolites and energy", "cellular response to growth factor stimulus" and "cellular response to toxic substance". On the other hand, we observed that females with cognitive deficits (postnatal-FLX) displayed selective alterations of the so-called "negative regulation of cell proliferation", "negative regulation of cellular component organization", "response to starvation", "process utilizing autophagic mechanism and organelle fission".

Going deeper, we studied which pathways were altered by the different manipulations (Fig. 5E), taking into account three different databases. Crossing the results obtained in each experimental group, we observed, as shown in Fig. 5C, that 14 pathways (such as, for example, "immune system", "cytokine signaling in immune system", "IL-17" and "TNF" signaling pathways) were altered regardless of the sex or the period of manipulation, while 4 ("mTOR signaling", "cellular response to external stimuli", "apoptosis" and "NOD-like receptor signaling pathway") were specific for the females of the postnatal-FLX group.

Finally, considering the modulation of specific genes, one-way ANOVA revealed a significant effect of the modulation in both males ($F_{2,20} = 4.25$, $p < 0.05$) and females ($F_{2,17} = 10.9$, $p < 0.001$) for *Dusp1* expression (Fig. 5A). Specifically, we observed that prenatal FLX led to increased level of *Dusp1* in both males and females compared to vehicle (males: +44%, $p < 0.05$; females: +297%, $p < 0.001$. Tukey's multiple comparison test).

Moreover, only in males we found that the manipulation led to significant effect in the expression of *Fkbp5* ($F_{2,21} = 8.93$, $p < 0.01$), *Gadd45β* ($F_{2,21} = 8.96$, $p < 0.01$), and of the proinflammatory cytokines *Il1β* ($F_{2,21} = 5.60$, $p < 0.05$) and *Tnfα* ($F_{2,21} = 9.40$, $p < 0.01$) (Fig. 5A). In detail, the Tukey's multiple comparison test revealed that both pre- and postnatal modulations were associated to increased expression of *Fkbp5* (prenatal-FLX: +42%, $p < 0.001$; postnatal-FLX: +33%, $p < 0.05$), *Gadd45β* (prenatal-FLX: +53%, $p < 0.01$; postnatal-FLX: +46%, $p < 0.01$) and of the proinflammatory cytokines *Il1β* (prenatal-FLX: +49%, $p < 0.05$; postnatal-FLX: +38%, $p < 0.05$) and *Tnfα* (prenatal-FLX: +111%, $p < 0.01$; postnatal-FLX: +110%, $p < 0.01$) in comparison to the vehicle group.

3.6. Perinatal FLX induces molecular changes in male and female adult rat brain

In order to investigate whether the changes observed in the periphery may mirror similar effects at the central level, molecular analyses were also performed in the prefrontal cortex (PFC), dorsal hippocampus (dHip) and ventral hippocampus (vHip).

As it is shown in Fig. 6A-C, in males, we observed a general reduction in the mRNA levels of the genes considered. Specifically, this reduction was more robust in PFC (Fig. 6A) and dHip (Fig. 6B) than in the vHip (Fig. 6C) and it was more relevant in the prenatal-FLX group, which

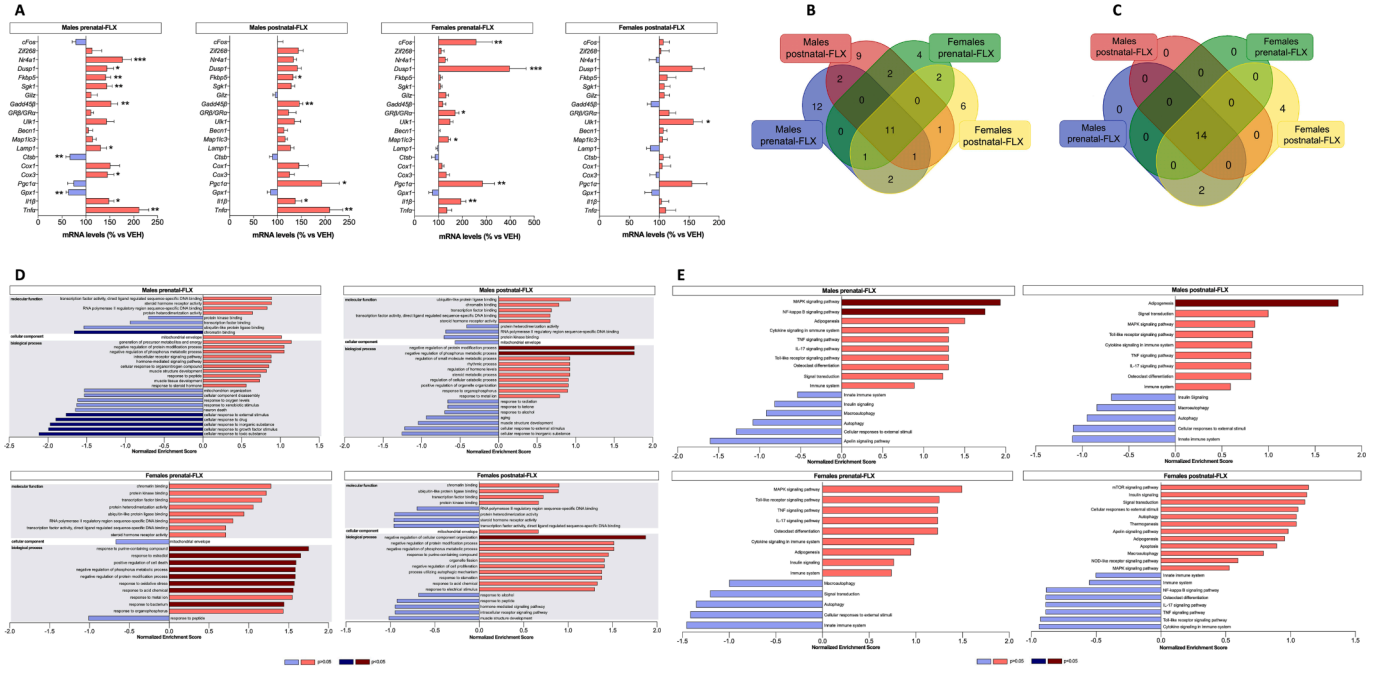

Fig. 5. Analysis of target mRNA levels in the blood of adult male and female rats exposed to perinatal FLX. Panel A shows the mRNA levels of the genes measured. Data are expressed as mean ± SEM of independent measures. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs vehicle (one-way ANOVA with Tukey multiple comparison test). Panel B shows the number of GO categories obtained by crossing the experimental groups. Panel C shows the number of pathways obtained by crossing the experimental groups. Panel D: GO function analysis divided in biological process, cellular component and molecular functions. Panel E: pathway analysis.

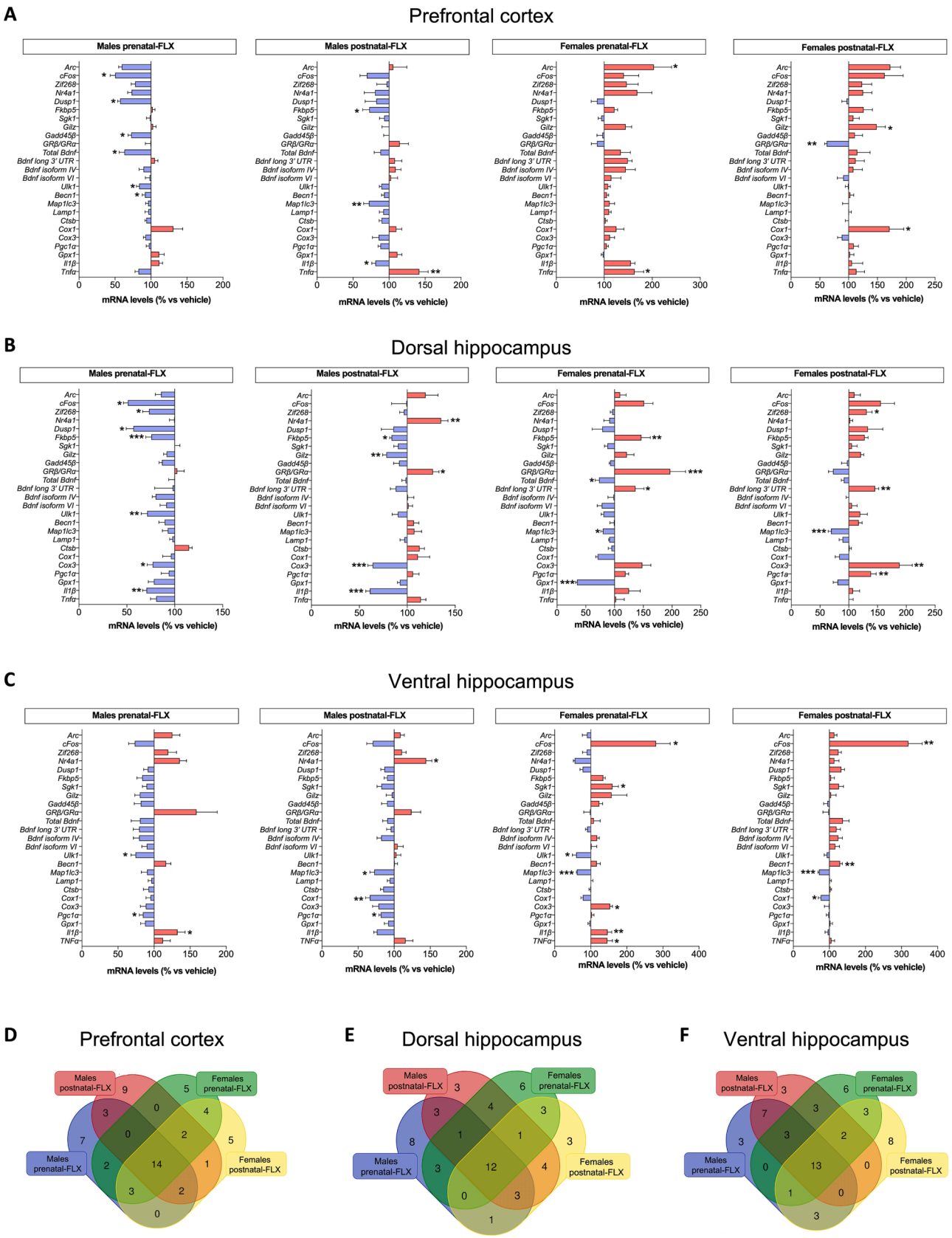

Fig. 6. Analysis of target mRNA levels in prefrontal cortex, in dorsal and in ventral hippocampus of adult male and female rats exposed to perinatal FLX. Panel A, B and C show the mRNA levels of the genes measured respectively in prefrontal cortex, in dorsal hippocampus and in ventral hippocampus. Data are expressed as mean ± SEM of independent measures. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs vehicle (one-way ANOVA with Tukey multiple comparison test). Panel D, E and F show the number of GO categories obtained by crossing the experimental groups respectively in prefrontal cortex, dorsal hippocampus and ventral hippocampus.

exhibited the anhedonic-like phenotype (Supplementary Table 9).

On the contrary, in female rats, we found an overall increased expression of the targets analyzed in comparison to vehicle due to the pharmacological administration, which was more robust in the two hippocampal subregions (Fig. 6B, C) than in the PFC (Fig. 6A) (Supplementary Table 10).

Through GO analysis (Supplementary Figs. 1,3,5), and similarly to what we observed in blood, we found common categories involved in both pre- and postnatal manipulation regardless of sex, while other categories were in common among the groups of the same sex and others resulted in being specific for the experimental groups.

In particular, 14, 12 and 13 were common to all the groups in PFC, dHip and vHip, respectively. Among these categories, 8 were modulated in all the brain regions and also in the blood ("mitochondrial envelope", "transcription factor activity", "direct ligand regulated sequence-specific DNA binding", "RNA polymerase II regulatory region sequence-specific DNA binding", "chromatin binding", "transcription factor binding", "protein kinase binding", "ubiquitin-like protein ligase binding", "protein heterodimerization activity").

Moreover, in PFC (Fig. 6D and Supplementary Table 12), 7 categories were modulated in males prenatal-FLX, including "aging" and "cellular response to growth factor stimulus", 9 in males postnatal-FLX, 5 in females prenatal-FLX, 5 in females postnatal-FLX, such as "cellular component disassembly", 3 were common among males and 4 among females.

In dHip (Fig. 6E and Supplementary Table 13) we found 8 categories specifically altered in males prenatal-FLX, including "aging", "cellular component disassembly" "response to steroid hormone", and "regulation of cellular catabolic process", 3 in males postnatal-FLX, 6 in females prenatal-FLX, 3 in females postnatal-FLX ("response to starvation", "neuron death" and "process utilizing autophagic mechanism"), 3 were shared by males and 3 by females.

In vHip (Fig. 6F and Supplementary Table 14) 3 categories were modified in males prenatal-FLX such as "developmental growth" and "cell growth", 3 in males postnatal-FLX, 6 in females prenatal-FLX, 8 in females postnatal-FLX including "response to starvation", "process utilizing autophagic mechanism", "behavior", and "cognition", 7 were in common among males and 3 among females.

Similarly to what we have done with blood, we performed the pathway analysis for each brain area and the results are outlined in the Supplementary materials (Supplementary Figs. 2, 4, 6). Interestingly, 10 pathways ("immune system", "toll-like receptor signaling pathway", "cytokine signaling in immune system", "IL-17 signaling pathway", "TNF signaling pathway", "macroautophagy", "cellular responses to external stimuli", "MAPK signaling pathway", "signal transduction", "osteoclast differentiation") were modulated in all groups, in the blood and the three brain regions considered.

Moreover, the animals that developed the anhedonic-like phenotype (male prenatal-FLX) showed an alteration of "apelin signaling" in PFC and "NF-kappa B signaling pathway" in vHip, while in the group that developed the cognitive impairment (females postnatal-FLX), we found alterations in "thermogenesis" (PFC), "apoptosis" (dHip), "NF-kappa B signaling pathway" (vHip and dHip), and "mTOR" and "NOD-like receptor" signaling pathways (in all the three areas).

4. Discussion

In this study, we observed that the consequences of transient modulation of the serotonergic system during perinatal life persist over time. Notably, we identified two different windows of vulnerability, with males being more sensitive to prenatal FLX and females being more susceptible to the early postnatal modulation. Moreover, we identified some specific potential biomarkers of the early-in-life manipulation of the serotonergic system that are common in both blood and brain.

According to previous preclinical and clinical studies (Chambers et al., 1996; Olivier et al., 2011; van den Hove et al., 2008), our results showed that rats treated with FLX during pregnancy deliver smaller litters probably because of the vasoconstrictive effect of serotonin on the umbilical arteries, which would, in turn, cause impairment of placental blood flow and disturbances in intrauterine development (Müller et al., 2013). We also observed that pups exposed to prenatal FLX weighed less at birth but recovered before weaning. On the other hand, in agreement with the preclinical study of Pinheiro et al., 2019, rats exposed to postnatal FLX gained less weight compared to the control group and, interestingly, they remained smaller into adulthood although SSRI exposure terminated at PND21. This finding could be related to the fact that the postnatal period is essential for the food intake programming in rodents (Pinheiro et al., 2019), but further investigations are needed to verify this hypothesis.

According to the role played by 5-HT in the maturation of specific brain regions related to emotional and cognitive behavior (Glover and Clinton, 2016; Brummelte et al., 2017), we found that perinatal administration of FLX leads to the development of pathological-like phenotypes that are different according to the period of exposure and the sex. Indeed, in line with the evidence showing the influence of sex on the development of psychiatric disorders (Altemus et al., 2014; May et al., 2019; Tesic et al., 2019), which becomes manifest in adulthood (Solmi et al., 2022), we observed that adult prenatal-FLX males developed an anhedonic-like phenotype, while adult postnatal-FLX females manifested cognitive deficits and less anxious behavior, which interestingly is also present in the prenatal-FLX group since adolescence.

Despite over the years several lines of research have focused on the behavioral effects of perinatal SSRIs exposure later in life, the results are often controversial (Hutchison et al., 2021; Ramsteijn et al., 2020). In particular, in line with the data presented here, some studies showed an association between prenatal SSRIs exposure and depressive-like behavior (Bhagya et al., 2015; Glover et al., 2015; Lisboa et al., 2007; Sprowles et al., 2017, 2016; Zohar et al., 2016), cognitive dysfunctions (Meurer et al., 2021; Sadegzadeh et al., 2020; Olivier et al., 2011; Linhares et al., 2022), and anxiety levels (Gemmel et al., 2019, 2018; Lisboa et al., 2007; Rayen et al., 2015; Rodriguez-Porcel et al., 2011). On the contrary, other studies have reported that prenatal or neonatal SSRIs exposure decreased or had no effect on depressive-like symptoms (Avitsur et al., 2016; Francis-Oliveira et al., 2013; Laureano-Melo et al., 2020; McAllister et al., 2012; Olivier et al., 2011; Sprowles et al., 2017), cognitive functions (Ansorge et al., 2004; Bairy et al., 2007; Sprowles et al., 2017; Meurer et al., 2021; Laureano-Melo et al., 2020; Kroeze et al., 2016; Svirsky et al., 2016) and anxiety-like behavior (Hanley et al., 2015; Hermansen et al., 2016; Matsumoto et al., 2016; Meyer et al., 2018) during adulthood. This discrepancy is probably due to the different experimental conditions employed in each study making a direct comparison and, consequently, the possibility of conclusions to be drawn difficult. For this reason, in our study, we attempted to consider several variables in the same cohort of rats thus allowing us to have the most comprehensive profile of the results).

Specifically, our protocol took into account the effects exerted by time-specific alterations of the serotonergic system at different ages in both sexes. Moreover, given the heterogeneous nature of symptoms affecting patients with psychiatric disorders, we evaluated the expression of several molecular markers in order to identify potential differences that might be useful, in association with the behavioral alterations, for the stratification in more homogeneous populations.

Indeed, our experiments have highlighted a complex biological picture following perinatal exposure to FLX. We observed that the pharmacological modulation of the serotonergic system leads to changes in blood, in the prefrontal cortex (PFC), in the dorsal hippocampus (dHip), and ventral hippocampus (vHip) regardless of sex or timing of exposure to FLX of 8 GO categories and 10 pathways. These changes were related to fundamental functions, such as mitochondrial, transcriptional and protein functions, but also immunity processes such as "immune system", "cytokine signaling in immune system", "IL-17" and "TNF" signaling pathways, supporting previous evidence showing that

serotonin alterations have a huge impact on the inflammatory system (Wu et al., 2019a, 2019b), which is indeed long-lasting.

Accordingly, the relationship between 5-HT and the inflammatory system has also been demonstrated for other diseases, such as irritable bowel syndrome (Najjar et al., 2023). In addition, recent evidence showed that drugs acting on the serotonergic system have anti-inflammatory effects in different pathological conditions, such as after myocardial infarction (Abdel-Hameed et al., 2023) and in multiple sclerosis (Stamoula et al., 2021).

Focusing on the experimental groups that developed pathological phenotypes, we observed specific modulations common in blood and brain. In particular, the results herein presented support the potential relationship between the anhedonic-like phenotype (males prenatal-FLX) and alterations regarding growth factors (Matsuno et al., 2022; Pisoni et al., 2018; Wu et al., 2019b), response to steroid hormone (Brivio et al., 2021a; Nowacki et al., 2020), apelin (Bullich et al., 2022) and NF-kappa B (Koo et al., 2010) signaling pathways. On the other hand, according to literature, cognitive impairment (females postnatal-FLX) seems to be associated with variations in autophagic processes (Tripathi et al., 2019; Zhai et al., 2018), NF-kappa B (Kaltschmidt and Kaltschmidt, 2015), NOD-like receptor (Tavolieri et al., 2020) and mTOR (Van Skike et al., 2020) signaling pathways, apoptosis (Itoh et al., 2013) and thermogenesis (Xiong et al., 2022). Moreover, we found alterations in this group in the response to starvation, which could be related to the lower body weight (Mottarlini et al., 2022).

In addition, we found changes specifically at the central level but not in the blood. Indeed, in males prenatal-FLX we found dysregulation in aging and developmental growth, in line with Han et al., 2021 and Whittle et al., 2014, while in females postnatal-FLX the results showed alterations in behavior and cognition reflecting their phenotype.

Interestingly, our results highlighted that some changes are specific for the groups that did not manifest any pathological-like phenotype, which could be related to behaviors different from those analyzed or to different times of onset.

Focusing on the effects of specific genes, we found that the upregulation of *Dusp1* expression in the blood of both male and female prenatal-FLX animals might be considered a stable index of the prenatal perturbation of the serotonergic system.

Moreover, we found that *Fkbp5* and *Gadd45β* mRNA levels were selectively increased in male rats exposed to perinatal FLX and this is extremely interesting since these genes have been previously associated with altered stress response and antidepressant treatments (Ising et al., 2019; Shen et al., 2022).

Interestingly, and in line with evidence of increased levels of pro-inflammatory markers in both the periphery and the brain of depressed patients (Black and Miller, 2015; Enache et al., 2019; Goldsmith et al., 2016), we observed that perinatal manipulation of the serotonergic system upregulated the expression of *Il1β* and *Tnfα* in the blood of male rats.

Accordingly, we previously demonstrated that genetic alteration of the serotonergic transmission induced an increase of inflammatory markers at the central level (Macchi et al., 2013), but, in this work, we made a step forward showing that inflammation is also modulated at the peripheral level suggesting that patients with altered expression of inflammatory markers in the blood might be good candidates to receive drugs acting on the 5-HT system.

The behavioral and molecular differences between sexes could be due to the sex-specific development of the serotonergic system and the fact that FLX can have a different effect in males and females (Brummelte et al., 2017). Indeed, it is well known that 5-HT has a crucial role in sexual differentiation via the hypothalamic-pituitary–gonadal axis (Jarzab and Döhler, 1984). Furthermore, the different 5-HT levels in the first stages of life in male and in female rodents (Connell et al., 2004) could justify the different time windows of susceptibility and the diverse pathological phenotypes.

Finally, it should be considered that this study has some limitations. First, one may argue that we primarily rely on gene expression to infer changes in function; however, the persistence of mRNA changes over time (i.e., from development throughout adulthood) is indeed suggestive of potentially functional effects. Moreover, we did not evaluate maternal behavior, and thereby we cannot exclude that part of these effects could be due to different maternal care. On the other hand, the mothers used in this study were naïve and, therefore, we can assume that FLX may have had limited and/or positive effects on their behavior at best. Furthermore, we did not measure FLX levels in the offspring but, based on other published work with similar experimental protocols (Ansorge et al., 2004; Capello et al., 2011; Kiryanova et al., 2016; Noorlander et al., 2008; Olivier et al., 2008), the drug administered to the mother effectively reaches the brain of the pups. Lastly, in this study we did not evaluate the estrous cycle of the female offspring during the behavioral tests and the collection of tissues.

5. Conclusion

The results presented in this study suggest that perinatal pharmacological manipulation of the serotonergic system leads to age- and sex-dependent effects indicating that males should be studied primarily for anhedonic-like symptoms whereas cognitive dysfunction should be first investigated in female rats. Moreover, we believe that the sex-specific molecular alterations in blood herein identified should be further studied as potential biomarkers related to specific symptoms to achieve earlier diagnosis and better patient stratification. Finally, the current study supports the idea that the pharmacological manipulation of the serotonergic system could be used as a tool to explore the role of serotonin during brain development and its involvement in the development of psychiatric disorders.

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Ethics approval

All procedures used in this study have conformed to the rules and principles of the 2010/63 European Communities Council Directive and were conducted in accordance with the authorization n. 472/2021-PR approved by the Italian Health Ministry in line with the Italian legislation in animal experimentation (DL 26/2014).

CRediT authorship contribution statement

Maria Teresa Gallo: Methodology, Data curation, Formal analysis, Writing – original draft. **Paola Brivio:** Conceptualization, Investigation, Methodology, Data curation, Formal analysis. **Beatrice Dolci:** Methodology. **Fabio Fumagalli:** Investigation. **Francesca Calabrese:** Conceptualization, Investigation, Formal analysis, Writing – original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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