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*Original Research*

# SERT-Deficient Mice Fed Western Diet Reveal Altered Metabolic and Pro-Inflammatory Responses of the Liver: A Link to Abnormal Behaviors

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## Abstract

**Background**: The inheritance of the short *SLC6A4* allele, encoding the serotonin transporter (SERT) in humans, increases susceptibility to neuropsychiatric and metabolic disorders, with aging and female sex further exacerbating these conditions. Both central and peripheral mechanisms of the compromised serotonin (5-HT) system play crucial roles in this context. Previous studies on SERT-deficient ($Sert^{-/-}$) mice, which model human SERT deficiency, have demonstrated emotional and metabolic disturbances, exacerbated by exposure to a high-fat Western diet (WD). Growing evidence suggests the significance of hepatic regulatory mechanisms in the neurobiology of central nervous system disorders, supporting the 'liver-brain' concept. However, the relationship between aberrant behavior and hepatic alterations under conditions of SERT deficiency remains poorly investigated. **Methods**: One-year-old female $Sert^{-/-}$ mice and their wild-type (WT) littermates were subjected to a control diet (CD) or the WD for a duration of three weeks. The WD had a higher caloric content and was characterized by an elevated saturated fat content (21%) compared to the CD (4.5%) and contained 0.2% cholesterol. Mice were evaluated for anxiety-like behavior, exploration and locomotor activity in the open field test, as well as glucose tolerance and histological indicators of hepatic steatosis. Hepatic pro-inflammatory and metabolism-related gene expression and markers of nitrosative stress, were analyzed utilizing real-time polymerase chain reaction (RT-PCR) and correlated with behavioral and histological outcomes. **Results**: In comparison to unchallenged mice, $Sert^{-/-}$/WD mutants, but not the WT/WD group, had increased locomotion and anxiety-like behavior, increased hepatic steatosis, and elevated expression of insulin receptor B and pro-inflammatory cytokines interleukin-1$\beta$ (*Il-1*$\beta$) and *Tnf*, as well as decreased expression of leptin receptor B. The two genotypes displayed distinct gene expression patterns of nitric oxide (NO)-related molecules inducible NO synthase (*iNos*) and arginase (*Arg2*), insulin receptor-related signaling factors: cluster of differentiation 36 (*Cd36*), ecto-nucleotide pyrophosphatase/phosphodiesterase (*Enpp*), protein tyrosine phosphatase N1 (*Ptpn1*), cytochrome P450 omega-hydroxylase 4A14 (*Cyp4a14*), acyl-CoA synthetase 1 (*Acsl1*) and phosphatase and tensin homolog (*Pten*). Furthermore, there were profound differences in correlations between molecular, histological, and behavioral measurements across the two genotypes. **Conclusions**: Our findings suggest that the genetic deficiency of SERT results in abnormal hepatic pro-inflammatory and metabolic adaptations in response to WD. The significant correlations observed between behavioral measures and pro-inflammatory and metabolic alterations in WD-fed mice suggest the importance of liver-brain interactions and their role in the aberrant behaviors exhibited by $Sert^{-/-}$ mutants. This study presents the first evidence that altered liver functions are associated with pathological behaviors arising from genetic SERT deficiency.

**Keywords:** serotonin transporter (SERT); $Sert^{-/-}$ mice; Western diet; aging; impulsive behavior; insulin receptor; leptin receptor; liver steatosis; nitrosative stress; pro-inflammatory cytokines

## 1. Background

Genetically determined reduction in serotonin transporter (SERT) function represents one of the few genetic risk factors whose contribution to the incidence of anxiety and depression remains significant across decades of research [1–4]. Diminished SERT expression can result from a polymorphism of the *SLC6A4* gene encoding SERT, wherein the so-called short (s) allele, a variant of the upstream regulatory region of *SLC6A4*, is associated with decreased SERT activity compared to the long (l) allele [1,5]. Individuals carrying the ss-allele exhibit higher morbidity of anxiety and depression precipitated by stress and adverse life events [1,6]. Among carriers of the short *SLC6A4* allele, females are diagnosed with depression at twice the rate of males [7]. Genetically reduced SERT function in s-allele carriers is also associated with type 2 diabetes and obesity [7,8]. In addition to female sex, advanced age, and preference for a high-calorie dietary pattern, such as the 'Western diet' (WD), which is rich in saturated fat and cholesterol, are further exacerbating factors for increased risk of metabolic and psychiatric disturbances, as well as other syndromes associated with the s-allele of *SLC6A4* [9]. While both central and peripheral mechanisms underlie morbidities related to genetic SERT deficits, few mechanistic studies have addressed their potential interconnection.

Clinical and mechanistic investigations conducted over recent decades have elucidated that elevated extracellular serotonin (5-HT) concentrations, resulting from SERT deficiency, constitute a critical pathogenic factor in the etiology of various syndromes associated with *SLC6A4* gene polymorphisms [10–13]. These alterations can be found in both the central nervous system and peripheral tissues, leading to dysregulation of diverse 5-HT receptor subclasses, molecular mediators of inflammation, and insulin receptor (IR)-mediated processes [10,12–17]. Among the peripheral organs, the liver is profoundly affected by SERT dysregulation and subsequent increases in 5-HT levels [16–18]. SERT is abundantly expressed in hepatic tissue; hepatocytes possess can transport and metabolize 5-HT, although they do not synthesize it [17]. 5-HT produced in the gastrointestinal tract and transported to the liver can exert a direct steatogenic effect and induce insulin resistance [18–20]. Consequently, the genetic absence of SERT in rodents can result in hepatosteatosis under normal conditions [16], and under highly caloric feeding regimens [11–15]; these pathological effects are more pronounced in females [21,22]. Furthermore, central and peripheral 'sterile' inflammation were observed in WD-fed Sert$^{-/-}$ rodents [11,13,22,23]. Hence, genetic SERT deficiency significantly affects numerous processes in the liver, which can be substantially exacerbated by a WD-like dietary challenge.

Accumulating evidence suggests the significance of hepatic regulatory mechanisms in the neurobiology of central nervous system (CNS) disorders. The concepts of 'liver-brain' and 'brain-liver axes' have gained general acceptance [24]. Pionnering research by Campbell *et al.* [25] demonstrated the crucial role of the liver in mediating CNS-driven sterile inflammation: in a rat model of brain injury, the anti-inflammatory effects of therapeutic agents were mediated through the attenuation of the hepatic chemokine response [25]. In a rat model of cerebral ischemia, anti-tumor necrosis factor (TNF) therapy reduced hepatic expression of chemokine ligand and TNF in spleen [26]. Elevated liver and spleen levels of interleukin-1$\beta$ (IL-1$\beta$) and TNF were associated with increased anxiety-like behavior and aggression in St3gal5$^{-/-}$ mutants [27] and in stressed mice exhibiting a depressive-like phenotype, anxiety-like behavior, and aggressive behavior [28].

Clinical observations align with translational findings [29,30]. Specifically, patients with chronic liver inflammation demonstrate sickness behavior, chronic fatigue, and depressive-like symptoms [31,32]. In individuals diagnosed with clinical depression, elevated levels of normal-range liver metabolic and peripheral inflammatory markers, including TNF and IL-1$\beta$, correlate positively with negative mood scores [31,33–35]. Concurrently, individuals experiencing depression exhibit a higher propensity for developing non-alcoholic fatty liver disease (NAFLD) compared to non-depressed individuals, independent of manifestations of diabetes and obesity [36,37]. Thus, impaired liver function and the biology of neuropsychiatric and neurological disorders are interrelated. While accumulating evidence indicates the significance of hepatic regulatory mechanisms in the neurobiology of CNS disorders, the relationship between aberrant behavior and liver alterations under conditions of genetic SERT deficiency remains poorly investigated.

In this study, we aimed to investigate the hepatic responses of Sert$^{-/-}$ mice subjected to a Western diet (WD) and to examine their metabolic, nitric oxide (NO)-related, and pro-inflammatory markers in relation to their altered emotionality, utilizing a previously established mouse WD paradigm [38–41]. In this model, mice fed a highly caloric diet enriched with saturated fat and cholesterol exhibit insulin resistance, glucose intolerance, hepatic steatosis, systemic inflammation, and aberrant emotionality, which are more pronounced in females [38–41]. SERT-deficient mice demonstrate exacerbated physiological and behavioral responses to the WD in comparison with standard laboratory mice [14,15,42]. Consequently, one-year-old Sert$^{-/-}$ females were administered a WD for three weeks and subsequently evaluated for glucose tolerance, hepatic lipid inclusion area, and behavioral parameters of locomotion, anxiety, and exploratory behavior.

Sert$^{-/-}$ mice are well documented to recapitulate human symptoms of elevated fear and anxiety, increased stress response, and other deficits that are reported in *ss-*

careers of the *SLC6A4* gene polymorphisms and are reminiscent of anxiety disorders and major depression [3,5]. Subsequently, we investigated the hepatic gene expression of IR isoform A (Ir-A) and IR isoform B (Ir-B), which are implicated in metabolic and NAFLD syndromes and exhibit distinct physiological roles [43]. We also studied the gene expression of leptin receptor (LR) isoforms A and B, which differentially regulate metabolic processes [44]. Furthermore, the gene expression of several signaling factors regulating IR functions and lipid metabolism was investigated. The expression of these factors is crucial in the mechanisms of type 2 diabetes, obesity, and related morbidities. We also studied the expression of cluster of differentiation 36 (*Cd36*), a receptor implicated in intracellular lipid storage, tissue uptake of fatty acids, insulin resistance, and pro-inflammatory responses [45]. Additionally, we examined gene expression of phosphatase and tensin homolog (*Pten*), a significant regulator of IR functions and glucose metabolism [46], and protein tyrosine phosphatase N1 (*Ptpn1*), which exhibits elevated levels in patients with type 2 diabetes [47]. We also aimed to investigate the expression of acyl-CoA synthetase 1 (*Acsl1*), which enhances inflammatory responses via Toll-like receptor 4 (TLR4) and pro-inflammatory cytokines and regulates triacylglycerol synthesis and fatty acid $\beta$-oxidation [48], as well as cytochrome P450 omega-hydroxylase 4A14 (*Cyp4a14*), a factor that governs insulin receptor-mediated signaling, glucose, and lipid metabolism [49].

Given the previously demonstrated role of TNF in liver-brain communication and emotional regulation [25,28,31,32], hepatic gene expression of this pro-inflammatory cytokine was examined. Additionally, the gene expression of cytokine *Il-1*$\beta$ was investigated, as it is implicated in liver-brain interactions and pathological behaviors in rodent models of neuropsychiatric pathologies and stress [50–53]. Considering that pro-inflammatory changes are associated with nitrosative stress [54,55], we further examined the gene expression of arginase (*Arg2*) and inducible NO synthase (*iNos*). The gene expression data were correlated with the histological analysis of liver steatosis and behavioral measures of emotionality that were evaluated in the battery of tests [14,42].

## 2. Materials and Methods

### *2.1 Animals*

Experiments were conducted utilizing 12-month-old Sert$^{-/-}$ female mice and wild-type littermates obtained from heterozygous mutants at the tenth generation of backcrosses with C57BL/6J mice. Breeding and genotyping were performed at the facilities of the University of Würzburg. Following the transportation of animals to the laboratories of C. Bernard University, mice were housed in groups of 4–5 per cage for a 2-week acclimatization period and throughout the study, under a reversed 12 h light-dark cycle (lights on: 21:00) with ad libitum access to food and water, under controlled laboratory conditions (22 $\pm$ 1 °C, 55% humidity). All experimental procedures were conducted in accordance with ARRIVE guidelines, the European Communities Council Directive for the Care and Use of Laboratory Animals (2010/63/EU) and approved by the local ethics committee of C. Bernard University (CBU 08RC2017).

### *2.2 Study Flow and Dietary Challenge*

Wild-type (WT) and Sert$^{-/-}$ mice were maintained on either a control diet (CD) or a high-fat, high-cholesterol diet ('Western diet', WD), supplied by Research Diet Inc. (New Brunswick, NJ, USA) as previously described [14,15,38,40]. For three weeks, experimental groups WT/CD (n = 6), Sert$^{-/-}$/CD (n = 8) were housed on a standard control diet (CD), WD was used in groups of WT and mutant mice: WT/WD (n = 7), Sert$^{-/-}$/WD (n = 8). The CD (D18071801, Research Diet Inc., New Brunswick, NJ, USA) had an energy content of 3.8 kcal/g, comprising 4.5% fat (0.6% saturated fat), whereas the WD (D11012302, Research Diet Inc., New Brunswick, NJ, USA) had an energy content of 4.6 kcal/g, comprising 21% fat (12% saturated fat) and 0.2% cholesterol. Details on general diet composition, energy content, specific nutrients, and the ingredients can be found in **Supplementary Tables 1A,B**).

Mice were weighed immediately prior to and following the period of dietary intervention; changes in body mass were calculated. This procedure preceded behavioral examination to rule out potential confounding factors in the measurement of their behavior. Throughout the course of the study, all mice were sequentially subjected to the open field test and glucose tolerance test, after which they were euthanized (*see below p.2.5*). Their livers were dissected and harvested for subsequent staining assay with Oil Red O for steatosis and gene expression analysis using reverse transcription polymerase chain reaction (RT-qPCR) (Fig. 1).

### *2.3 Open Field Test*

The open field model was utilized to examine locomotor and anxiety-like behavior in mice. The experiment was conducted during the active (dark) period of the animals' light cycle (09:00–21:00), as described elsewhere [56]. The open field apparatus comprised four square arenas (40 cm $\times$ 40 cm), enabling simultaneous observation of four animals (Technosmart, Rome, Italy). The mice were carefully positioned at the corner of the open field arena, and their actions were recorded on video for a duration of 5 min under moderate lighting conditions of 25 Lux. The rodents' behavior was examined in two distinct areas: the central region (a 20 cm $\times$ 20 cm square) and the peripheral zone (the remaining portion of the box). The behavioral analysis was conducted offline, employing both manual methods and the VideoTrack software (VideoTrack 3.10, ViewPoint Behavior Technology, Civrieux, France). The total distance traversed, number of rearing, duration of freezing

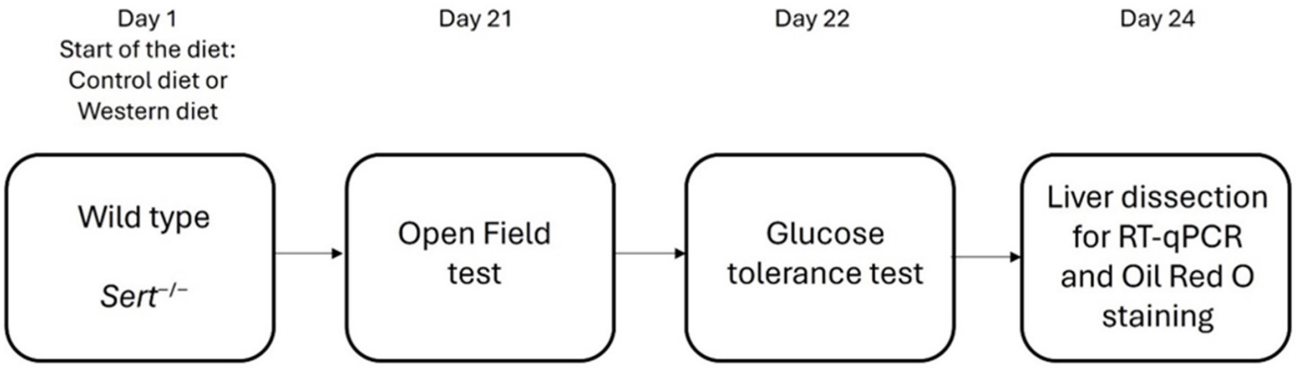


**Fig. 1. Experimental design.** Following a three-week period of housing on control diet (CD) or Western diet (WD), on day 21, mice were subjected to the open field test. The next day 22, mice were examined using a glucose tolerance test. On day 24, the mice were euthanized, and the liver was dissected for subsequent RNA isolation and quantitative reverse transcription polymerase chain reaction (RT-qPCR) assay and histological analysis of hepatosteatosis utilizing Oil Red O staining.

behavior, the number and duration of grooming events, time spent while in normal/contracted posture were recorded in the central and peripheral areas of the open field arena, additionally, time spent in the center, periphery, and corners and the mean distance from the walls was measured. For all groups, data were presented in absolute values, and for each WD-fed group, they were additionally normalized to the respective genotype fed with CD.

### *2.4 Glucose Tolerance Test*

The oral glucose tolerance test was administered to evaluate glucose tolerance in the experimental groups; the test was conducted as previously described [41]. All mouse groups underwent an overnight fasting period of eighteen hours, commencing at 16:00. On the subsequent day, mice were administered a glucose solution (2 g/kg, 1.8 g/L) via gavage following the fasting period. Blood samples were collected from the tail vein prior to glucose administration (0 min), and at every 15 min for two hours post-glucose administration. The OneTouch UltraEasy glucometer and test strips (LifeScan OneTouch, Dubai, UAE) were utilized to determine blood glucose concentrations.

### *2.5 Euthanasia and Tissue Dissection*

For euthanasia, in accordance with ARRIVE guidelines, mice were briefly exposed to $CO_2$ (15%) and subsequently subjected to isoflurane (5%) inhalation as described elsewhere [41]. Specifically, mice were placed into a $CO_2$-containing chamber and exposed to isoflurane inhalation until the loss of reflexes. All animals were transcardially perfused with ice-cold 10 mL of 0.9% NaCl, and their livers were subsequently dissected. Portions of the livers were isolated and immediately frozen at –80 °C on dry ice for gene expression analysis. The remaining portions of the livers were stored in neutral buffered formaldehyde 10% v/v (SurgiPath Europe Ltd., Bretton, Peterborough, Cambridgeshire, UK) until further use for Oil Red O staining.

### *2.6 RNA Extraction and RT-qPCR*

To examine the gene expression of specific genes of interest (*Ir-A*, *Ir-B*, *Lr-A*, *Lr-B*, *Tnf*, *Il-1β*, *iNos*, *Arg2*, *Acsl1*, *Enpp1*, *Ptpn1*, *Pten*, *Cd36*), we conducted messenger ribonucleic acid (mRNA) extraction and complementary deoxyribonucleic acid (cDNA) synthesis following previously described methods [40]. The RNA extraction process involved utilizing precisely microdissected snap-frozen liver samples [40]. Synthetically designed amplicons were employed to generate standard curves. The specific primers, developed through primer design, are available in **Supplementary Table 2**. The genes of interest and the reference gene glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) were analyzed using RT-qPCR. RNA extraction was performed with the RNeasy Mini Kit (Qiagen, Venlo, The Netherlands). The High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA, USA) was utilized to synthesize first-strand cDNA, with 1 µg of total RNA converted to cDNA. For the analysis, the SYBR Green PCR Master Mix and QuantStudio 7 Flex Real-Time qPCR System (both from Applied Biosystems, Thermo Fisher Scientific, MA, USA) were used. The Pfaffl method was employed to normalize the RT-qPCR assay results, with data expressed in Ct values, in accordance with previously described protocols [40]. Results are presented as fold changes in expression relative to the mean expression levels in WT/CD, which served as the control. Additionally, for each genotype administered WD, the data were normalized against its corresponding CD-fed group.

### *2.7 Oil Red O Staining*

Oil Red O staining was conducted according to a previously established protocol [41]. Briefly, liver smaterial was initially fixed overnight using 10% formaldehyde (Thermo Fisher Scientific, Breda, The Netherlands), followed by a 3.5-hour wash in water. The samples were subsequently placed in 12.5% gelatin (Thermo Fisher Scien-

tific, Breda, The Netherlands) for 4 h at 37 °C, and then transferred to 20% gelatin overnight at 37 °C. Subsequently, utilizing a cryostat (Cryostat CM1860, Leica Biosystems Nussloch GmbH, Germany) at a temperature of –17 °C, the tissue was sectioned and five 10-µm sections were obtained for each animal. The sections were then briefly washed with distilled water and dehydrated in 60% isopropanol. Oil Red O (Thermo Fisher Scientific, Breda, The Netherlands) solution (0.05 µg/mL in 98% isopropanol) was applied to stain the sections for 10 min. Histological sections were mounted in glycerol-gelatin containing 6.25% gelatin (w/w), 0.045% camphor (w/w), and 56.22% glycerol (w/w) and covered with coverslips. Histological analysis of the stained sections was performed using LAS X software (Leica Biosystems, Nussloch GmbH, Germany) on a light microscope DMI4000 (Leica Biosystems, Nussloch GmbH, Germany) at 20× and 40× magnification with a DFC490 camera (Leica Biosystems, Nussloch GmbH, Germany). The total area of lipid inclusions in the liver was scored for each section as described elsewhere [39,41]. Data were averaged for each animal.

### *2.8 Statistics*

GraphPad Prism version 8.01 (Graphpad Prism, San Diego, CA, USA) was utilized for data analysis. Initially, the Shapiro-Wilk normality test was applied to assess the distribution of all quantitative datasets. Subsequently, since the data exhibited normal distribution, two-way analysis of variances (ANOVA) was conducted, followed by Tukey's multiple comparison test for post hoc analysis. Data from each WD-fed genotype were standardized against their respective CD groups and compared to 100%. For the examination of normalized data, *t*-tests or one-sample *t*-tests were employed. Correlation analysis was performed using linear regression. Statistical significance was established at $p < 0.05$. Results are expressed as mean ± SEM.

## 3. Results

### *3.1 Elevated Body Mass and Impaired Glucose Tolerance in Sert$^{-/-}$ Mice Exposed to WD*

A significant diet effect was observed in body weight changes between the onset of the diet and the end of the experiment, with differences among groups as follows: WT/CD: 0.38 ± 0.13; Sert$^{-/-}$/CD: 4.21 ± 0.31; WT/WD: 0.25 ± 0.72; Sert$^{-/-}$/WD: 4.5 ± 0.4 ($F = 70.46$, $p < 0.0001$, two-way ANOVA). Body weight was significantly increased after three weeks of WD feeding in both the WT ($p = 0.002$, post hoc Tukey's test) and Sert$^{-/-}$ groups ($p < 0.0001$). No significant genotype effect or genotype × diet interaction was found ($F = 0.0278$, $p = 0.869$ and $F = 0.181$, $p = 0.674$, respectively). A significant diet effect was also observed in the difference between basal glucose levels and peak blood glucose at 15 min after glucose administration (WT/CD: 5.38 ± 1.65; Sert$^{-/-}$/CD: 11.78 ± 2.78; WT/WD: 13.57 ± 2.67; Sert$^{-/-}$/WD: 15.92 ± 3.19, $F = 4.921$, $p = 0.0413$). No significant genotype effect or genotype × diet interaction was revealed ($F = 2.483$, $p = 0.1347$ and $F = 0.5313$, $p = 0.4766$, respectively).

### *3.2 Western Diet Increased Total Area of lipid Inclusions in Liver of Sert$^{-/-}$ and WT Mice*

Two-way ANOVA showed a significant diet effect on the total area of lipid inclusions in the liver ($F = 53.64$, $p < 0.0001$), no significant genotype effect or genotype × diet interaction was found ($F = 0.0003$, $p = 0.986$ and $F = 0.211$, $p = 0.651$, respectively). Lipid inclusion area was significantly elevated in both WD-fed WT and Sert$^{-/-}$ mice ($p = 0.0008$ and $p = 0.0001$, respectively, post hoc Tukey's test; Fig. 2A). When normalized to the corresponding CD groups and set to 100%, the lipid inclusion area was significantly larger in both WT/WD and Sert$^{-/-}$/WD groups ($p = 0.0037$ and $p = 0.0019$, respectively, one-sample *t*-test). Additionally, the normalized lipid inclusion area was significantly larger in Sert$^{-/-}$/WD mice compared to WT/WD mice ($p = 0.0104$, *t*-test; Fig. 2A). Overall, these findings indicate that Sert$^{-/-}$ mice exhibit more pronounced histological changes in WD-induced liver steatosis compared to WT mice (Fig. 2A–E).

### *3.3 Western Diet and SERT Deficiency Affected Behavioral Parameters in the Open Field*

A significant genotype effect was observed for the number of rearings in the center of the open field ($F = 11.27$, $p = 0.0025$, two-way ANOVA; Fig. 3A); there was no significant diet effect or genotype × diet interaction ($F = 2.237$, $p = 0.1473$ and $F = 2.514$, $p = 0.1254$, respectively). The number of center rearings was significantly lower in the WT/WD group compared to the WT/CD group ($p = 0.0108$, Tukey's test). In the WT/WD group, the normalized number of rearings in the center was significantly decreased ($p = 0.0440$, one-sample *t*-test). For rearings in the periphery, a significant genotype effect was observed ($F = 4.475$, $p = 0.0445$), while diet and genotype × diet interaction were non-significant ($F = 0.328$, $p = 0.572$ and $F = 0.429$, $p = 0.518$, respectively; Fig. 3B). For the total number of rearings, a significant genotype effect was found ($F = 8.021$, $p = 0.0090$), with no significant effect of diet or genotype × diet interaction ($F = 0.554$, $p = 0.464$ and $F = 0.727$, $p = 0.402$, respectively; Fig. 3C).

A significant genotype effect was found for the duration of freezing ($F = 7.08$, $p = 0.0134$, two-way ANOVA), although no significant differences were observed between groups (Fig. 3D). For time spent with normal body length, a significant genotype effect was observed ($F = 4.446$, $p = 0.0452$); no significant effects of diet or genotype × diet interaction were found ($F = 0.382$, $p = 0.542$ and $F = 0.804$, $p = 0.378$, respectively; Fig. 3E). For time spent in a contracted posture, two-way ANOVA did not reveal significant genotype or diet effects, nor an interaction effect ($F = 0.659$, $p = 0.425$; $F = 0.813$, $p = 0.376$; and $F = 2.231$, $p = 0.148$,

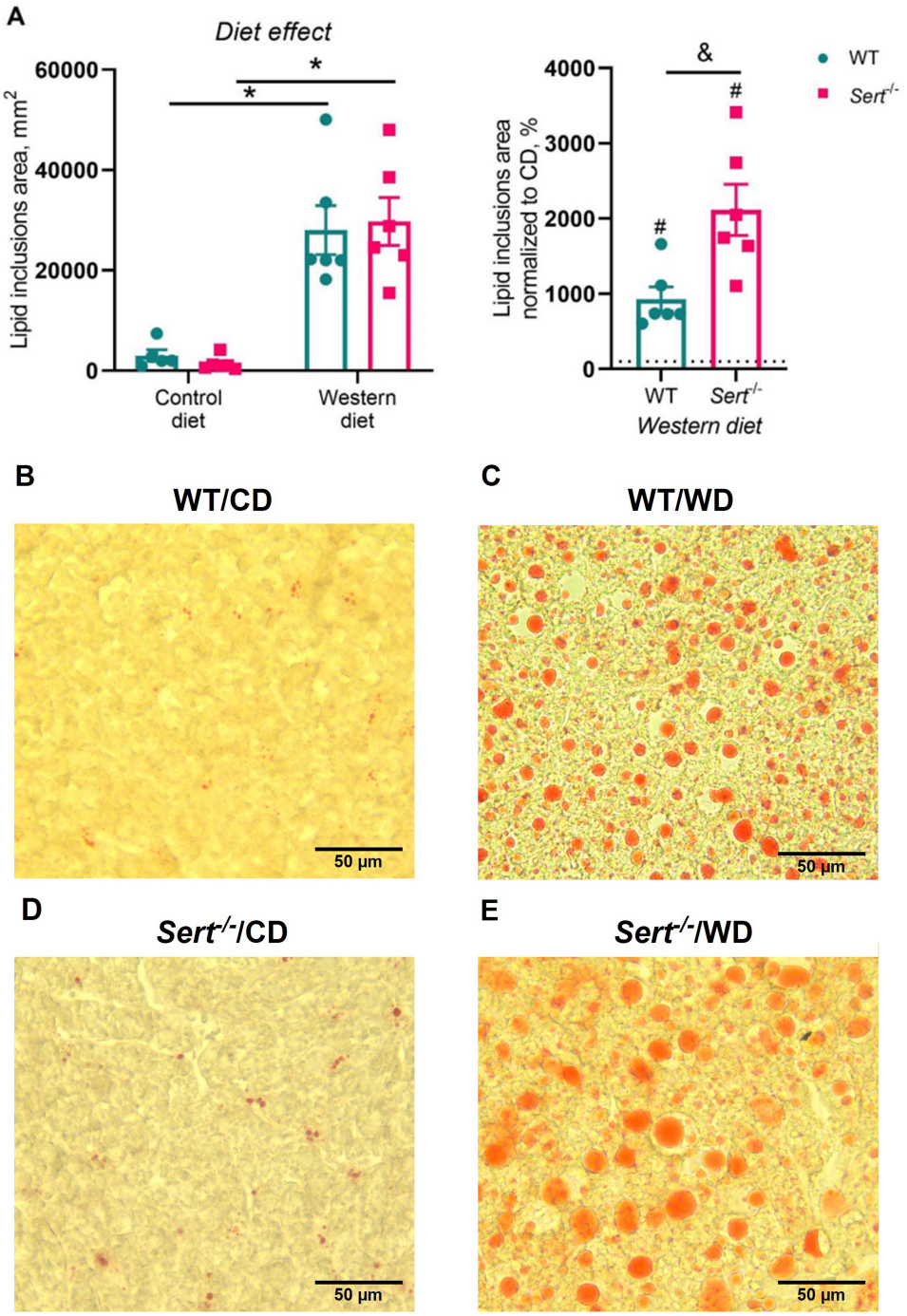


**Fig. 2. Effects of WD and the genetic absence of SERT on lipid inclusions in the liver.** (A) WD exposure significantly increased the total area of lipid inclusions in the livers of both WT and Sert$^{-/-}$ mice. (B) Sert$^{-/-}$ mice fed a WD displayed a significantly larger lipid inclusion area than WT mice housed on the WD. (C–E) Representative microphotographs of liver sections from each experimental group, stained with Oil Red O, show lipid inclusions A — *$p < 0.05$, two-way ANOVA, post-hoc Tukey's test; B — #$p < 0.05$ vs. 100%, one-sample *t*-test; &$p < 0.05$, unpaired *t*-test. Scale bar = 50 µm. CD, control diet; WD, Western diet; WT, wild types; Sert$^{-/-}$, SERT knockout; SERT, serotonin transporter. 6 mice per group were used. All data are mean ± SEM.

respectively). However, Sert$^{-/-}$/WD mice showed significantly less time in this posture than the WT/WD group when normalized to respective CD groups ($p = 0.0376$, *t*-test; Fig. 3F). No significant effects of genotype, diet, or their interaction were found for the total distance traveled (F = 2.174, $p = 0.153$; F = 0.145, $p = 0.707$; and F = 1.767, $p = 0.196$, respectively). In Sert$^{-/-}$/WD mice, the normalized total distance traveled was significantly elevated when

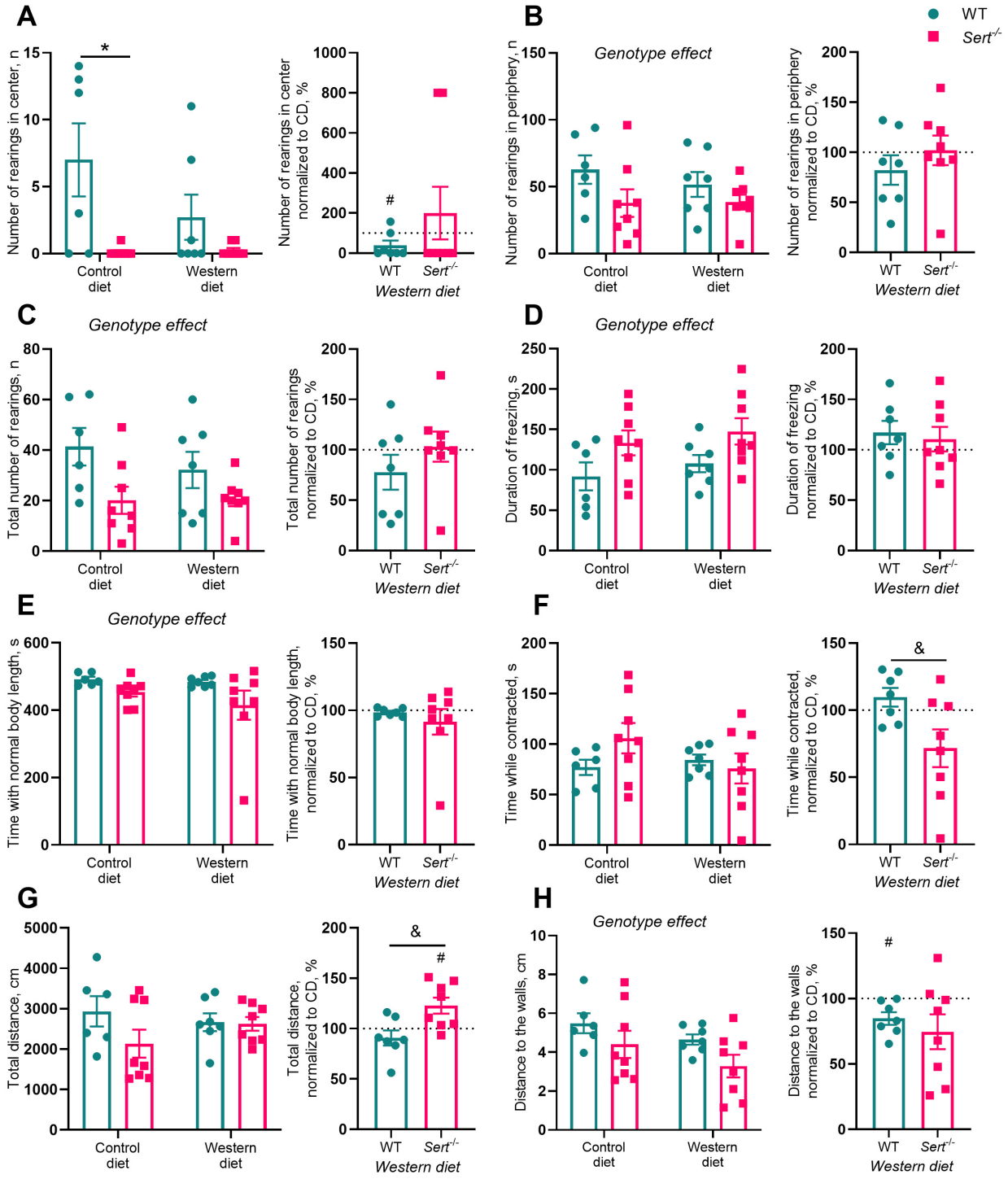


**Fig. 3. Effects of WD and the genetic absence of SERT on the open field locomotion, rearing and fear-related behaviors.** (A) The WT/WD group exhibited significantly fewer rearings in the center of the open field compared to WT/CD mice, the normalized number of rearings in the center was significantly lowered. (B) No significant group differences were observed in the number of rearings in the periphery. (C) Total number of rearings showed no significant differences between groups. (D) Duration of freezing, (E) time spent with normal body length, and (F) time spent in a contracted posture were not significantly different between the groups. The latter measure was significantly decreased in Sert$^{-/-}$/WD mice. (G) In Sert$^{-/-}$/WD mice, the normalized total distance traveled was significantly increased vs. WT/WD group and 100%. (H) In WT/WD mice, the normalized distance to the walls was significantly shortened. *$p < 0.05$, Two-way ANOVA and Tukey's test. #$p < 0.05$ vs. 100%, one-sample $t$-test, &$p < 0.05$, unpaired $t$-test. CD, control diet; WD, Western diet; WT, wild types; *Sert*$^{-/-}$, SERT knockout (SERT, serotonin transporter, WT/CD group, n = 6, WT-WD group, n = 7, *Sert*$^{-/-}$/CD group, n = 8, *Sert*$^{-/-}$/WD group, n = 8). All data are mean ± SEM.

compared with 100% and with that measure in WT/WD mice ($p$ = 0.0233, one-sample $t$-test, and $p$ = 0.0119, $t$-test, respectively; Fig. 3G). A significant genotype effect was observed for the distance to the walls (F = 4.713, $p$ = 0.0396; Fig. 3H). A trend toward a diet effect on wall distance was found (F = 2.978, $p$ = 0.0967), while no genotype $\times$ diet interaction effect was shown (F = 0.062, $p$ = 0.805). The WT/WD group had a significantly lowered normalized distance to the walls ($p$ = 0.0195, one-sample $t$-test); Sert$^{-/-}$/WD mice showed a similar trend ($p$ = 0.0977).

Genotype, diet, and genotype $\times$ diet interaction did not significantly affect the number of grooming events in the center of the open field (F = 0.0056, $p$ = 0.802; F = 0.383, $p$ = 0.542; and F = 0.044, $p$ = 0.835, respectively, two-way ANOVA). The WT/WD group exhibited a significant decrease in the normalized number of grooming events in the center ($p$ = 0.0428, one-sample $t$-test; Fig. 4A). No significant effects of genotype, diet, or their interaction were found for the total duration of grooming in the center (F = 0.128, $p$ = 0.724; F = 0.948, $p$ = 0.339; and F = 0.269, $p$ = 0.608, respectively; Fig. 4B). In the periphery, a significant genotype effect was observed on the number of grooming events (F = 20.42, $p$ = 0.0001), along with a significant genotype $\times$ diet interaction (F = 4.534, $p$ = 0.0433). In the Sert$^{-/-}$/WD group, the normalized number of grooming events in the periphery was significantly decreased ($p$ = 0.0062, one-sample $t$-test) and lower than that in WT/WD mice ($p$ = 0.0428, $t$-test; Fig. 4C). Additionally, the WT/WD group showed a significantly higher number of grooming events in the periphery compared to the WT/CD group ($p$ = 0.0006, Tukey's test). Trend for significant genotype effect was found for the duration of grooming events in the periphery (F = 3.097, $p$ = 0.0907, respectively); there was no significant diet effect or genotype $\times$ diet interaction for this measure (F = 2.517, $p$ = 0.125 and F = 0.941, $p$ = 0.341, respectively; Fig. 4D).

No significant effects of genotype, diet, or genotype $\times$ diet interaction were observed for the time spent in the center of the open field or in the periphery (F = 2.527, $p$ = 0.125; F = 0.209, $p$ = 0.651; and F = 1.043, $p$ = 0.317, respectively; Fig. 4E,F). No significant effects of genotype, diet, or their interaction were found for time spent in the corners (F = 0.798, $p$ = 0.380; F = 0.001, $p$ = 0.9965; and F = 1.908, $p$ = 0.179, respectively). Normalized time in the corners in Sert$^{-/-}$/WD mice was non-significantly shorter than in WT/WD mice ($p$ = 0.056, $t$-test). This parameter was non-significantly increased in the WT/WD group compared to 100% ($p$ = 0.0521, one-sample $t$-test; Fig. 4G).

### *3.4 Western Diet and SERT Deficiency Altered Liver Gene Expression of Leptin Receptors, Insulin Receptor B and Pro-Inflammatory Cytokines*

A significant genotype effect (F = 16.98, $p$ = 0.0005), but not diet effect or their interaction (F = 0.422, $p$ = 0.523 and F = 2.027, $p$ = 0.169, respectively), was observed for *Il-1β* expression; this measure was higher in WT/WD mice than in WT/CD mice ($p$ = 0.0065, Tukey's test, Fig. 5A). In the WT/WD group, the normalized *Il-1β* expression was significantly higher than in Sert$^{-/-}$/WD group ($p$ = 0.0389, $t$-test). A significant diet effect was found for *Tnf* expression levels (F = 4.561, $p$ = 0.0441). No significant genotype effect or genotype $\times$ diet interaction was observed for this measure (F = 0.832, $p$ = 0.371 and F = 2.328, $p$ = 0.141, respectively). *Tnf* expression was higher in the Sert$^{-/-}$/WD group than in the Sert$^{-/-}$/CD mice ($p$ = 0.0459, Tukey's test; Fig. 5B). Sert$^{-/-}$/WD animals revealed significantly elevated normalized *Tnf* expression ($p$ = 0.0314, one-sample $t$-test); the WT/WD group showed a trend for such an increase ($p$ = 0.0848, one-sample $t$-test).

Genotype had a significant effect on the mRNA level of *Arg2* (F = 13.52, two-way ANOVA, $p$ = 0.0013). mRNA level of *Arg2* was significantly higher in the Sert$^{-/-}$/WD group than in the WT/WD mice ($p$ = 0.0284, Fig. 5C). No significant diet effect or genotype $\times$ diet interaction was found for this measure (F = 1.914, $p$ = 0.18; and F = 0.39, $p$ = 0.539, respectively). Similarly, the genotype effect, but not the diet effect or their interaction, was significant for *iNos* expression (F = 5.55, $p$ = 0.0287; F = 1.919, $p$ = 0.182; and F = 2.585, $p$ = 0.124, respectively). There was a trend for an increase in *iNos* mRNA concentration in Sert$^{-/-}$/WD mice as compared to both the Sert$^{-/-}$/CD and WT/WD groups ($p$ = 0.0996 and $p$ = 0.0636, respectively, Tukey's test, Fig. 5D). A trend for an increase in normalized *iNos* mRNA concentration was observed in the Sert$^{-/-}$/WD group ($p$ = 0.0694, one sample $t$-test).

A significant diet effect (F = 27.34, $p < 0.0001$), genotype effect (F = 28.85, $p < 0.0001$), and significant genotype $\times$ diet interaction (F = 6.764, $p$ = 0.016, two-way ANOVA) were shown for *Lr-A* expression. mRNA level of *Lr-A* was significantly decreased in both WT/WD and Sert$^{-/-}$/CD compared to that in WT/CD ($p$ = 0.0002 and $p < 0.0001$, respectively, Tukey's test, Fig. 5E). The normalized mRNA *Lr-A* level was significantly lowered in both the WT/WD and Sert$^{-/-}$/WD groups (both $p < 0.0001$, one-sample $t$-test). Significant effects of genotype (F = 19.32, $p$ = 0.0002) and diet (F = 18.53, $p$ = 0.0003), but not their interaction (F = 0.335, $p$ = 0.568), were found for *Lr-B* expression. This measure was significantly lower in Sert$^{-/-}$/CD mice than that in WT/CD mice ($p$ = 0.0218, Tukey's test; Fig. 5F). A trend for lowered expression of *Lr-B* was shown for WT/WD compared to WT/CD ($p$ = 0.0898). The normalized mRNA *Lr-B* concentration was significantly reduced in the Sert$^{-/-}$/WD group compared to 100% ($p < 0.0001$, one-sample $t$-test).

No significant genotype or diet effects were found for *Ir-A* expression (F = 0.016, $p$ = 0.9003 and F = 0.941, $p$ = 0.342, respectively; two-way ANOVA). A trend was revealed for the genotype $\times$ diet interaction for *Ir-A* expression (F = 3.104, $p$ = 0.0914). In the WT/WD group, normalized *Ir-A* expression was significantly decreased com-

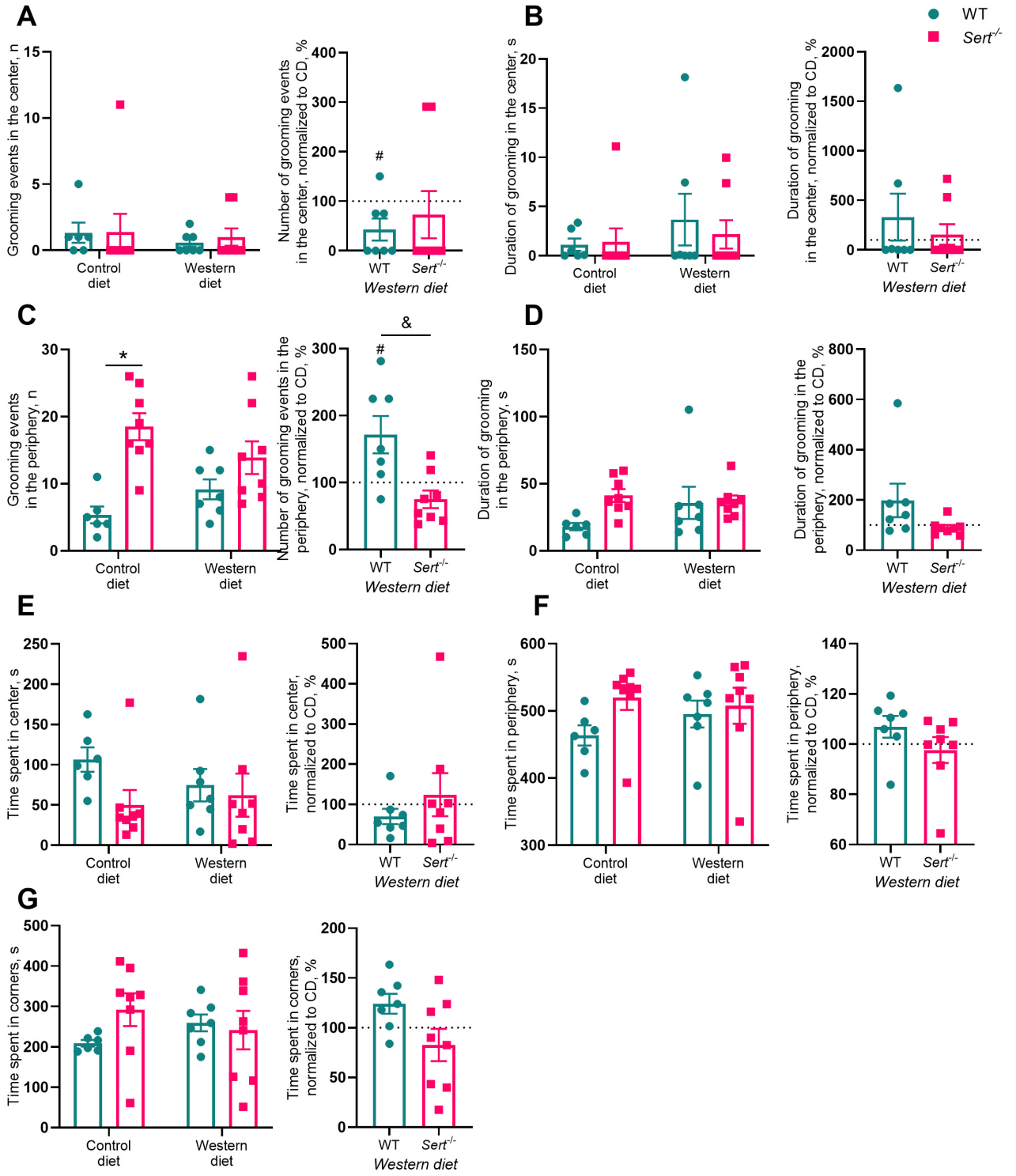


**Fig. 4. Effects of WD and the genetic absence of SERT on the grooming and anxiety-like behavior in the open field.** (A) In the WT/WD group, the normalized number of grooming events in the center was significantly reduced. (B) No significant group differences were found in the duration of grooming in the center. (C) The number of peripheral grooming events was significantly higher in the Sert$^{-/-}$/WD group than in the WT/CD group. In the Sert$^{-/-}$/WD group, normalized number of grooming events was significantly lower than in WT/WD mice. This measure was significantly higher in the WT/WD group. No significant group differences were found for (D) duration of grooming in the periphery, (E) time spent in the center, (F) time spent in the periphery, or (G) time spent in the corners. *$p < 0.05$, Two-way ANOVA and Tukey's test. #$p < 0.05$ vs. 100%, one-sample $t$-test, &$p < 0.05$, unpaired $t$-test. CD, control diet; WD, Western diet; WT, wild types; Sert$^{-/-}$, SERT knockout (WT/CD group, n = 6, WT-WD group, n = 7, Sert$^{-/-}$/CD group, n = 8, Sert$^{-/-}$/WD group, n = 8). All data are mean ± SEM.

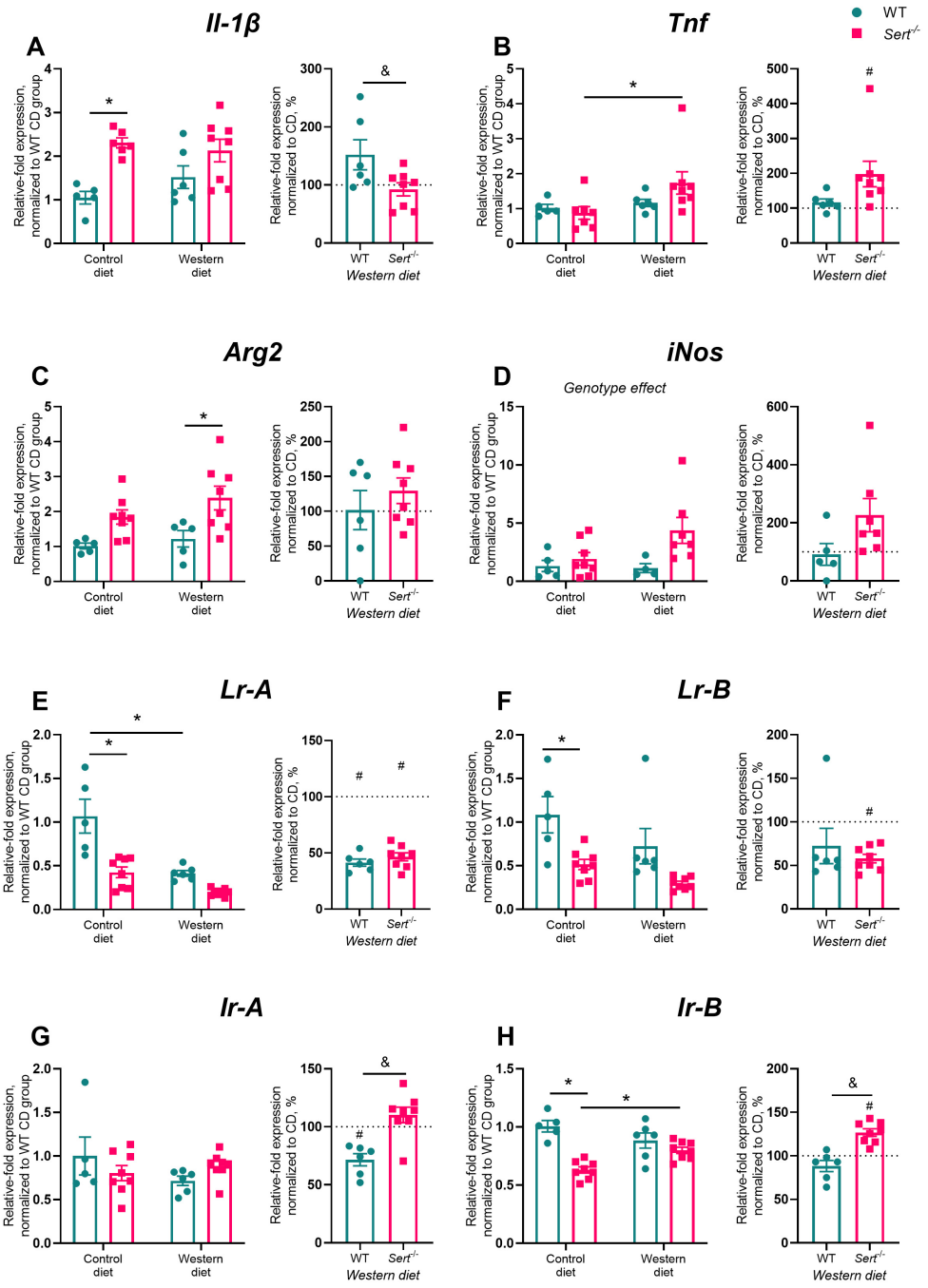


**Fig. 5. Gene expression of pro-inflammatory cytokines, leptin, and insulin receptors in the liver of WT and Sert$^{-/-}$ mice.** (A) *Il-1β* expression in the Sert$^{-/-}$/CD group was significantly increased compared to that in WT/CD mice. WD-induced expression of *Il-1β* was significantly higher in WT mice than in Sert$^{-/-}$ mice. (B) WD challenge significantly increased *Tnf* expression in Sert$^{-/-}$ mice but not in WT mice. (C) *Arg2* expression was significantly elevated in the Sert$^{-/-}$/WD group compared to that in WT/WD mice; no significant differences in *Arg2* expression were found between the CD groups. (D) No significant group differences in *iNos* expression were observed. (E) *Lr-A* expression was significantly lower in Sert$^{-/-}$/CD mice than in WT/CD mice. WD feeding significantly decreased *Lr-A* expression in WT mice but not in Sert$^{-/-}$ mice. Normalized *Lr-A* expression was significantly reduced. (F) *Lr-B* expression in Sert$^{-/-}$/CD mice was significantly lower than in the WT/CD group. Normalized *Lr-B* expression in Sert$^{-/-}$/WD group was significantly decreased. (G) Normalized *Ir-A* expression was significantly augmented in the WT group but not in Sert$^{-/-}$ mice. (H) *Ir-B* expression was significantly lower in Sert$^{-/-}$/CD mice than in WT/CD and than in Sert$^{-/-}$/WD mice. Normalized *Ir-B* expression was significantly augmented in Sert$^{-/-}$ mice but not in the WT group. *$p < 0.05$, two-way ANOVA, post-hoc Tukey's test; #$p < 0.05$ vs. 100%, one-sample *t*-test; &$p < 0.05$, unpaired *t*-test. CD, control diet; WD, Western diet; WT, wild-type; Sert$^{-/-}$, SERT knockout (WT/CD group, n = 6; WT/WD group, n = 7; Sert$^{-/-}$/CD group, n = 8; Sert$^{-/-}$/WD group, n = 8). All data are mean ± SEM.

pared to 100% ($p$ = 0.0003, one-sample $t$-test), and to Sert$^{-/-}$/WD group ($p$ = 0.0011, $t$-test; Fig. 5G). For *Ir-B* expression, a significant genotype effect (F = 30.79, $p$ < 0.0001), but not diet effect (F = 0.308, $p$ = 0.584), was observed; there was a significant genotype × diet interaction (F = 12.33, $p$ = 0.0019). *Ir-B* expression was significantly lower in Sert$^{-/-}$/CD mice than in WT/CD mice ($p$ < 0.0001, Tukey's test, Fig. 5H). *Ir-B* expression was significantly higher in the Sert$^{-/-}$/WD group than in the Sert$^{-/-}$/CD group ($p$ = 0.0196). Sert$^{-/-}$/WD group showed significantly elevated expression of normalized *Ir-B* ($p$ = 0.0005, one-sample $t$-test) and the WT/WD group ($p$ = 0.0002, $t$-test).

### *3.5 Effects of Western Diet and SERT Deficiency on Liver Expression of Transcription and Signaling Factors*

We found a trend for the effect of genotype on *Cd36* expression (F = 3.14, $p$ = 0.0924) and no significant genotype or genotype × diet interaction for this parameter (F = 2.65, $p$ = 0.12; and F = 0.437, $p$ = 0.517, respectively). In the Sert$^{-/-}$/WD group, the normalized *Cd36* expression was significantly decreased ($p$ = 0.0099, one-sample $t$-test, Fig. 6A). No significant effects of genotype, diet, or their interaction were revealed for the expression of *Acsl1* (F = 0.306, $p$ = 0.586; F = 0.297, $p$ = 0.592; and F = 0.695, $p$ = 0.418, respectively). WT/WD group demonstrated significantly decreased expression of normalized *Acsl1* mRNA level ($p$ = 0.0108, one-sample $t$-test, Fig. 6B).

A trend for significant interaction between genotype and diet was observed for *Cyp4a14* expression (F = 3.281, $p$ = 0.0859, two-way ANOVA); no significant effect of genotype or diet was observed (F = 0.047, $p$ = 0.83 and F = 0.272, $p$ = 0.608, respectively). In the WT/WD group, normalized *Cyp4a14* expression was decreased ($p$ = 0.0036, one-sample $t$-test, Fig. 6C), and there was a trend for reduced *Cyp4a14* expression in this group compared to Sert$^{-/-}$/WD group ($p$ = 0.0595, $t$-test). No significant effects of genotype, diet, or their interaction were observed on *Pten* expression (F = 0.414, $p$ = 0.528; F = 1.254, $p$ = 0.277; and F = 1.363, $p$ = 0.256, respectively) or *Ptpn1* expression (F = 1.285, $p$ = 0.273; F = 1.211, $p$ = 0.286; and F = 0.0036, $p$ = 0.953, respectively). In the WT/WD group, the normalized *Pten* expression was significantly reduced ($p$ = 0.0099, one-sample $t$-test, Fig. 6D). In this group, normalized *Ptpn1* expression was significantly decreased compared to 100% ($p$ = 0.0018, one-sample $t$-test; Fig. 6F). A significant diet effect was found on *Enpp1* expression (F = 4.824, $p$ = 0.0407), and no significant effects of genotype or genotype × diet interaction were demonstrated for this parameter (F = 0.0045, $p$ = 834 and F = 1.3, $p$ = 0.268, respectively). In the WT/WD group, normalized *Enpp1* expression was significantly decreased ($p$ = 0.0027, one-sample $t$-test; Fig. 6E).

Significant changes of gene expression of WT and Sert$^{-/-}$ mice fed CD or WD are summarized in Table 1.

**Table 1. Alterations of gene expression in the liver of CD- and WD-fed WT and Sert$^{-/-}$ mice.**

| Gene expression | Sert$^{-/-}$ vs. WT | WT/WD vs. WT/CD | Sert$^{-/-}$/WD vs. Sert$^{-/-}$/CD |
|---|---|---|---|
| *Il-1β* | ↑ | ↑ | - |
| *Tnf* | - | - | ↑ |
| *Arg2* | ↑ | - | - |
| *iNos* | ↑ | - | - |
| *Lr-A* | ↓ | ↓ | ↓ |
| *Lr-B* | ↓ | - | ↓ |
| *Ir-A* | - | ↓ | - |
| *Ir-B* | ↓ | - | ↑ |
| *Cd36* | ↑ | - | ↓ |
| *Acsl1* | - | ↓ | - |
| *Cyp4a14* | - | ↓ | - |
| *Pten* | - | ↓ | - |
| *Enpp1* | - | ↓ | - |
| *Ptpn1* | - | ↓ | - |

↑, significant increase of gene expression; ↓, significant decrease of gene expression; -, no significant group differences.

### *3.6 Correlations between Behavioral Parameters in the Open Field, Expression of Leptin Receptors, Pro-Inflammatory Cytokines, Signaling Factors, and Lipid Inclusions Area*

Correlation analysis was performed between the lipid inclusion area and the expression of genes encoding IR and LR, pro-inflammatory molecules, and signaling factors in the experimental groups of mice. For the graphical representation of significant correlations of the measured readouts with the lipid inclusion area (see Fig. 7), the statistical values of these correlations can be found in Table 2. Mirror changes in these correlations were observed in WT and Sert$^{-/-}$ mice in many cases. For example, highly significant correlations of inflammation and nitrosative stress parameters, *Tnf* and *iNos* expression, and the area of lipid inclusions were observed for Sert$^{-/-}$ mutants, but not for WD mice (Fig. 7A,B). *Ir-B* expression was positively correlated with the lipid inclusion area only in the mutants (Fig. 7C). Expression of both *Lr-A* and *Ir-B* isoforms showed negative correlations with lipid inclusion areas in both genotypes, with borderline significance (Fig. 7D,E). The expression of the signaling factors *Pten* and *Enpp1* was correlated with the area of lipid inclusions in the liver in the WT group, but not in Sert$^{-/-}$ mice (Fig. 7F,G).

In WT mice, but not in mutants, the lipid inclusion area correlated positively with the time spent with normal body length, and correlated negatively with the number of grooming events displayed in the periphery (Fig. 7H,I). Unlike the WT group, Sert$^{-/-}$ mice displayed a strong negative correlation between *Il-1β* expression in the liver and time spent in the center of the open field, and a positive correlation between time spent in the periphery. Sert$^{-/-}$ mu-

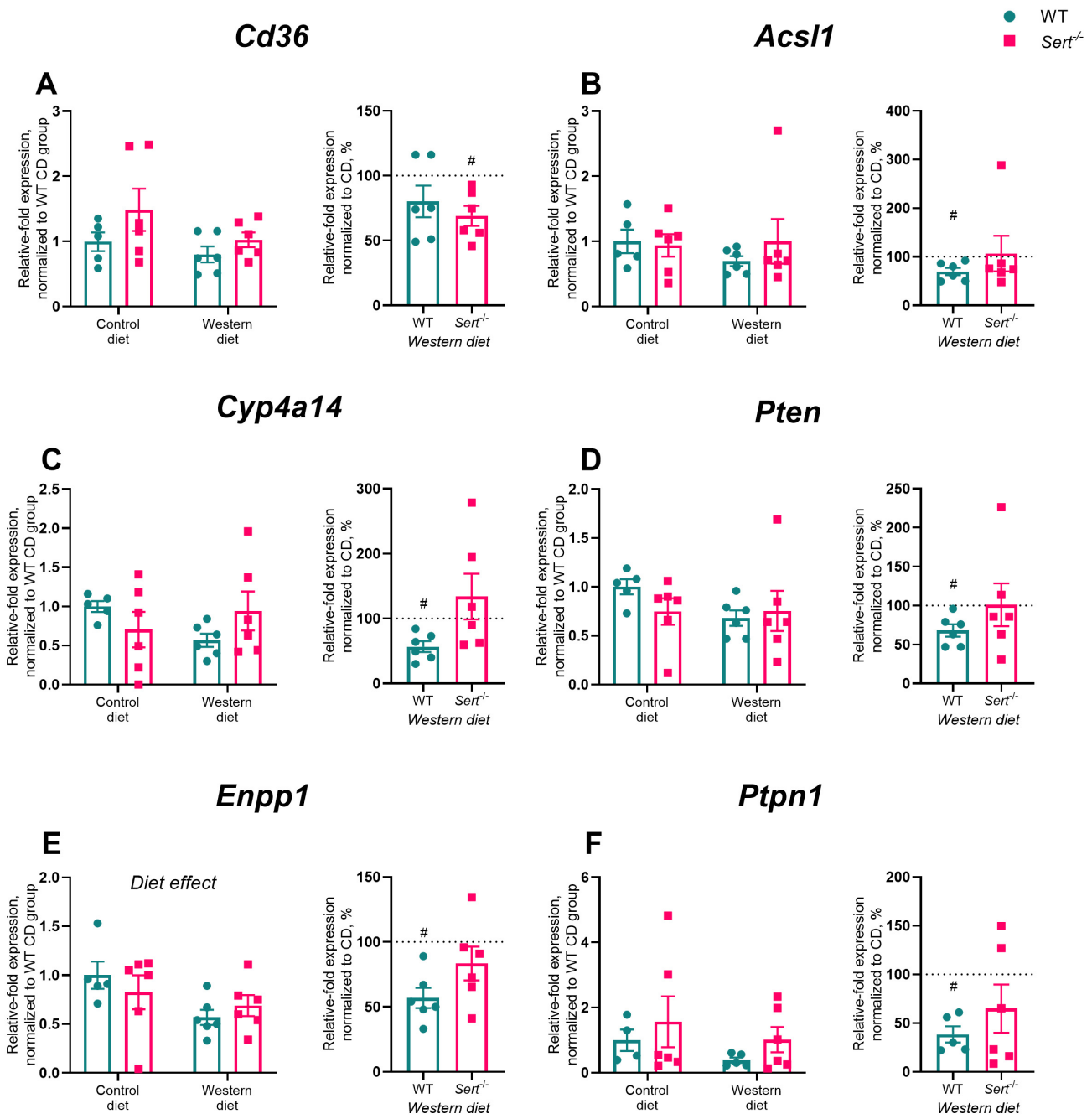


**Fig. 6. Gene expression of transcription and signaling factors in WT and Sert$^{-/-}$ mice fed WD.** (A) There was a significant decrease in normalized *Cd36* expression in Sert$^{-/-}$ WD animals but not in the WT/WD group. (B–F) Normalized expression of *Acsl1*, *Cyp4a14*, *Pten*, *Ptpn1*, and *Enpp1* was significantly lowered in WT/WD group, but not in the Sert$^{-/-}$/WD group. #$p < 0.05$ vs. 100%, one-sample *t*-test. CD, control diet; WD, Western diet; WT, wild types; Sert$^{-/-}$, SERT knockout (WT/CD group, n = 6, WT/WD group, n = 7, Sert$^{-/-}$/CD group, n = 8, Sert$^{-/-}$/WD group, n = 8). All data are mean ± SEM.

tants also showed positive correlations between the gene expression of *Arg2* and *iNos* and the total number of rearings and the number of rearings in the center, which are manifestations of behavioral hyperactivity. In the WT group, the expression of *Arg2* in the liver was negatively correlated with time spent in the center and positively correlated with time spent in the periphery and at the corners, measures of anxiety-like behavior.

In Sert$^{-/-}$ animals, but not in WT mice, *Lr-A* expression positively correlated with the total number of rearings and negatively correlated with the number of rearings in the periphery and measures of hyperactivity in mice; however, in WT mice, *Lr-A* expression positively correlated with grooming duration in the periphery. In Sert$^{-/-}$ mutants, *Lr-B* expression was positively correlated with the mean distance to the wall. Negative correlations were found

**Table 2. Correlation between lipid inclusions area, expression of LR, IR, pro-inflammatory and signaling factors and parameters of emotionality of CD- and WD-fed WT and Sert$^{-/-}$ mice.**

| Parameter | WT | Sert$^{-/-}$ |
|---|---|---|
| Correlation with liver steatosis | | |
| *Tnf* & Lipid inclusions area | ns | $p = 0.0157$, r = 0.676 |
| *iNos* & Lipid inclusions area | ns | $p = 0.0225$, r = 0.649 |
| *Ir-B* & Lipid inclusions area | ns | $p = 0.0003$, r = 0.859 |
| *Lr-B* & Lipid inclusions area | ns | $p = 0.0069$, r = –0.731 |
| *Lr-A* & Lipid inclusions area | $p = 0.0165$, r = –0.699 | ns |
| *Pten* & Lipid inclusions area | $p = 0.0085$, r = –0.745 | ns |
| *Enpp1* & Lipid inclusions area | $p = 0.0254$, r = –0.666 | ns |
| Time with normal body length & Lipid inclusions area | $p = 0.0419$, r = –0.619 | ns |
| Grooming events in the periphery & Lipid inclusions area | $p = 0.059$, r = 0.767 | ns |
| Correlation with expression of inflammatory and nitrosative stress markers | | |
| Time spent in the center & *Il-1β* | ns | $p = 0.0066$, r = –0.734 |
| Time spent in the periphery & *Il-1β* | ns | $p = 0.0066$, r = 0.734 |
| Number of rearings in the center & *iNos* | ns | $p = 0.0165$, r = 0.673 |
| Time spent in the center & *Arg2* | $p = 0.0021$, r = –0.874 | ns |
| Time spent in the periphery & *Arg2* | $p = 0.0021$, r = 0.874 | ns |
| Time spent in the corners & *Arg2* | $p = 0.0004$, r = 0.923 | ns |
| Correlation with leptin and insulin receptors expression | | |
| Number of rearingss in periphery & *Lr-A* | ns | $p = 0.0268$, r = –0.634 |
| Total number of rearings & *Lr-A* | ns | $p = 0.0064$, r = 0.736 |
| Time spent in the corners & *Ir-B* | ns | $p = 0.0441$, r = –0.589 |
| Number of rears in the center & *Ir-A* | ns | $p = 0.037$, r = 0.605 |
| Total number of rears & *Lr-B* | $p = 0.0165$, r = –0.219 | ns |
| Grooming events in the periphery & *Lr-B* | $p = 0.0228$, r = –0.674 | ns |
| Distance from the walls & *Ir-A* | $p = 0.0011$, r = 0.709 | ns |
| Duration of freezing & *Lr-B* | $p = 0.0479$, r = 0.607 | $p = 0.0175$, r = 0.668 |
| Correlation with expression of signaling factors | | |
| Number of rears in the center & *Acsl1* | ns | $p = 0.0451$, r = –0.613 |
| Time spent with normal body length & *Acsl1* | ns | $p = 0.0036$, r = 0.767 |
| Time spent while contracted & *Acsl1* | ns | $p = 0.0218$, r = –0.651 |
| Grooming events in the center & *Acsl1* | ns | $p = 0.0021$, r = –0.639 |
| Duration of freezing & *Pten* | ns | $p = 0.0194$, r = –0.66 |
| Duration of freezing & *Enpp1* | ns | $p = 0.0054$, r = –0.746 |
| Time spent with normal body length & *Cyp4a14* | ns | $p = 0.0272$, r = –0.633 |
| Distance from the walls & *Acsl1* | $p = 0.0015$, r = 0.692 | ns |
| Grooming events in the periphery & *Cyp4a14* | $p = 0.0303$, r = –0.715 | ns |
| Grooming duration in the periphery & *Cyp4a14* | $p = 0.0271$, r = –0.725 | ns |

*p*, r, statistical values of Pearson correlation; ns, non-significant. LR, leptin receptor; IR, insulin receptor.

between *Lr-B* expression in WT mice and the total number of rearing and grooming events in the periphery. Both genotypes showed significant positive correlations between *Lr-B* expression and the total duration of freezing, a measure of anxiety-like and fear behavior. In mutants, but not in WT mice, the expression of *Ir-A* correlated positively with the number of rearings in the center, and *Ir-B* correlated negatively with the time spent at the corners. A positive correlation between *Ir-A* expression and the distance to the walls was observed in WT mice but not in mutants.

Gene expression of *Acsl1* significantly correlated with parameters of anxiety-like behavior and impulsivity in Sert$^{-/-}$ mice, but not in WT mice. Specifically, in Sert$^{-/-}$ mice, a positive correlation was observed for the time spent with a normal body length, and a negative correlation was found for the time while contracted, as well as for the number of rearings and grooming events in the center of the open field. In WT mice, *Acsl1* expression correlated positively with the distance to the walls, another measure of anxiety-like behavior. *Cyp4a14* expression was negatively correlated with both grooming events in the center and grooming duration in the periphery and parameters of anxiety behavior in Sert$^{-/-}$ mice, whereas in the WT group, there was a negative correlation between the time spent with normal body length and *Cyp4a14* expression. Significant correlations between *Enpp1* and *Pten* expression were revealed

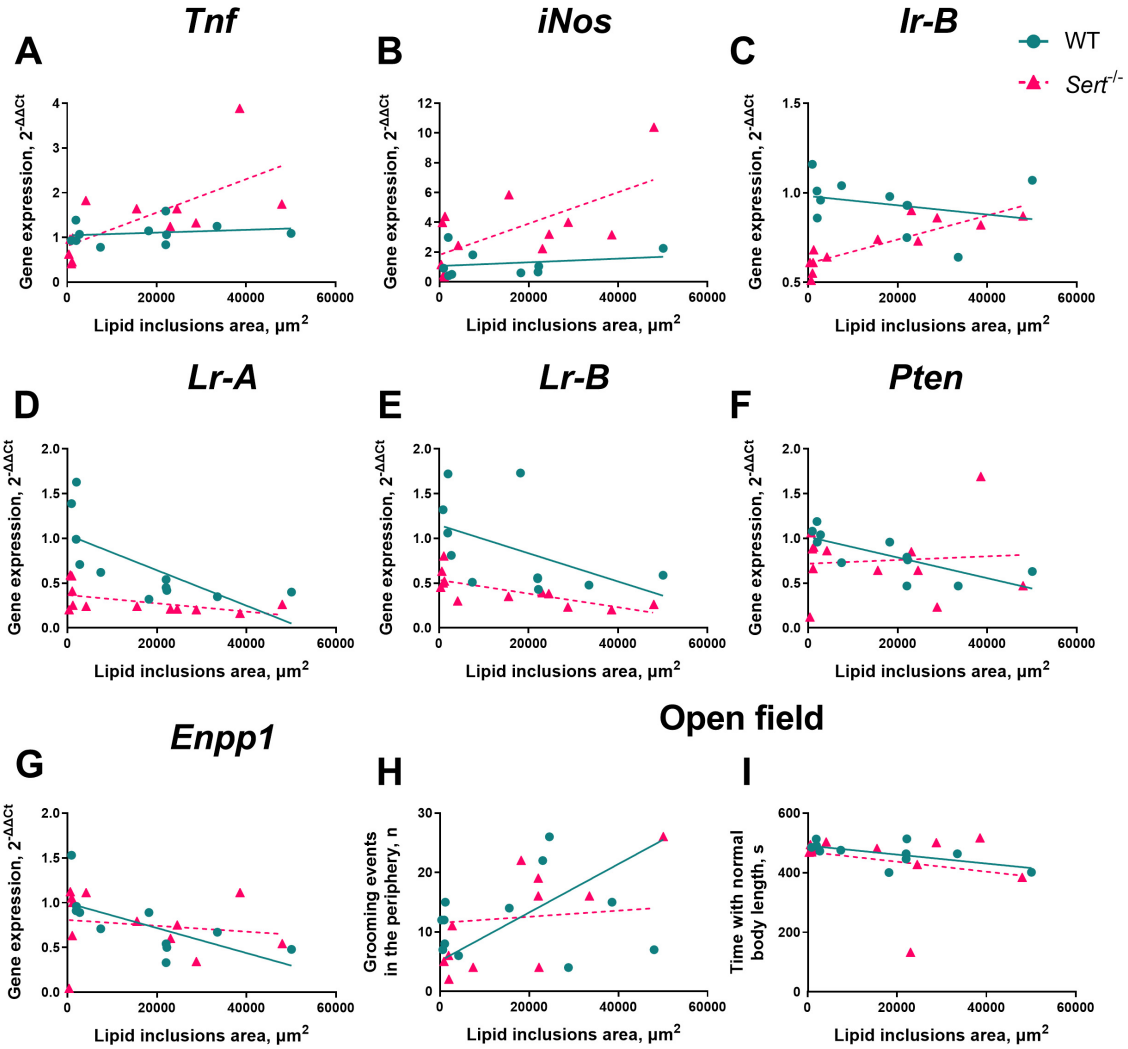


**Fig. 7. Correlation between lipid inclusion area and hepatic gene expression.** In Sert$^{-/-}$ mice, significant positive correlations were found between the lipid inclusion area and (A) *Tnf* expression, (B) *iNos* expression, and (C) *Ir-B* expression; a negative correlation was found with (E) *Lr-B* expression. In WT mice, negative correlations were found between (D) *Lr-A* expression, (F) *Pten* expression, (G) *Enpp1* expression, and (I) time with normal body length. (H) A positive correlation was found between the number of grooming events on the periphery (see Table 2 for *p* and r values).

only in mutant mice; both measures correlated negatively with the duration of freezing, a measure of anxiety-like and fear behaviors.

## 4. Discussion

Consistent with previously reported results, we found that three weeks of WD-exposure in aged female mice impaired glucose tolerance and led to body weight gain. In line with other studies using high-calorie diets and SERT-deficient rodents [11–15,42], our findings revealed strong effects of WD challenge on the area of lipid inclusions in the liver of Sert$^{-/-}$ mutants. Specifically, compared to the respective non-challenged groups, Sert$^{-/-}$ mice exhibited a 2.5-fold greater increase in this parameter than WT animals.

### *4.1 Genotype Differences in Anxiety-Like and Locomotor Behaviors in Naive and WD-exposed Groups*

Unchallenged Sert$^{-/-}$ mice exhibited decreased rearing scores, indicating suppressed novelty exploration—a sign of depressive-like behavior and an augmented stress response [56]. In comparison to naive animals, WT mice fed with WD showed a significant decrease in central exploration activity, while WD-fed Sert$^{-/-}$ mutants displayed opposing behavioral trends, including increased total distance crossed, indicating hyperactivity. SERT-deficient mice also spent a longer duration of freezing and exhibited a shorter distance to the wall—both signs of elevated anxiety—aligning with altered body length parameters, which were reflected in changes in the time spent in a freezing posture. Additionally, WD-exposed WT mice, but not mutants, showed an increased duration of grooming.

Thus, while both WD exposure and genetic SERT deficiency in rodents can induce behavioral changes associated with hyperactivity, anxiety, and depression [14,15,57,58], WD-exposed Sert$^{-/-}$ mice exhibited more profound alterations, often opposite to the behavioral traits of WT littermates. Interestingly, in WT mice, but not in mutants, the lipid inclusion area correlated with behavioral signs of anxiety, such as time spent with normal body length and grooming scores in the periphery.

### *4.2 Changes in Hepatic Gene Expression and their Correlations with Liver Steatosis and Behavior*

At the molecular level, we report, for the first time, that while hepatic expression of *Il-1*$\beta$ was significantly increased in the liver of naive Sert$^{-/-}$ animals compared to WT littermates, it was not significantly altered by WD exposure. In contrast, WD-fed WT mice exhibited a sharp increase in *Il-1*$\beta$ gene expression. Remarkably, while there was no significant correlation between hepatic *Il-1*$\beta$ gene expression and any of the studied behaviors in WT controls, Sert$^{-/-}$ mice showed a strong correlation between *Il-1*$\beta$ expression and time spent in the center versus the periphery—behavioral indicators of impulsivity and hyperactivity [40,59]. These findings support the role of IL-1$\beta$ in regulating behavior under conditions of stress and inflammation [28,51,60–62]. Additionally, IL-1$\beta$ is a key regulator of lipid metabolism [63–65].

We observed altered hepatic *Tnf* gene expression in both naive and Western diet (WD)-challenged Sert$^{-/-}$ mice, consistent with previously reported *Tnf* overexpression in the serum of naive Sert$^{-/-}$ mice [50]. Naive Sert$^{-/-}$ mice also exhibit elevated levels of other inflammatory markers in various tissues [23,50]. Specifically, elevated levels of pro-inflammatory cytokines TNF and IL-6, and cytokine-induced neutrophil chemoattractant 1 (CINC-1), cytokine-induced neutrophil chemoattractant 3 (CINC-3) and macrophage inflammatory protein (MIP-1$\alpha$) were observed in the plasma of Sert$^{-/-}$ mice [50]. A recent study by Hoch *et al*. [13] has demonstrated that young naive Sert$^{-/-}$ mice exhibit monocytosis associated with elevated levels of pro-inflammatory Ly6C+ monocytes, a condition exacerbated by WD exposure, which was not displayed by wild type mice. Furthermore, this study has shown that total leukocyte counts, macrophages, B cells, and T cells were increased in white adipose tissue of Sert$^{-/-}$ mice compared to controls [13].

Notably, while hepatic *Tnf* expression remained unchanged in naive mutants, WD exposure induced a significant upregulation in this group—an effect absent in WT mice. Although *Tnf* gene expression did not significantly correlate with emotionality measures, it was strongly associated with the extent of lipid inclusions in the livers of Sert$^{-/-}$ mice, a relationship not observed in WT littermates. This finding suggests that upregulated TNF may play a causative role in the hepatosteatosis seen in Sert$^{-/-}$ mice, potentially interlinked with the genetic deficit in SERT [65–67]. Collectively, our findings highlight distinct roles for hepatic IL-1$\beta$ and TNF in the context of SERT deficiency.

Studies have demonstrated that hepatic nitrosative stress is associated with the upregulation of pro-inflammatory cytokines and the exposure of rodents to highly caloric diets [68,69]. In our study, the gene expression of *Arg2* was upregulated in the livers of Sert$^{-/-}$ mice subjected to WD, while remaining unaltered in the WT group. The upregulation of Arg2 has been implicated in the mechanisms underlying obesity and diabetes [54,55,70,71]. Consistent with the observations for iNOS, Sert$^{-/-}$ mice, but not the WT group, exhibited significant correlations between the gene expression of *Arg2* and manifestations of behavioral hyperactivity. In the WT group, the gene expression of *Arg2* in the liver correlated with measures of anxiety-like behavior. Consequently, in the two genotypes, Arg2 appears to play differential roles in emotional regulation, suggesting a potential association with genetic SERT deficiency.

Similarly, *iNos* gene expression was elevated in the livers of Sert$^{-/-}$ mice exposed to WD, while remaining unaltered in the WT group. This observation aligns with previous studies demonstrating increased hepatic NO production and overexpression of the gene encoding iNOS in obese mice and rats maintained on a high-fat diet [54,55,72]. In the present study, *iNos* gene expression in the liver correlated with the lipid inclusion area of Sert$^{-/-}$ mice, but not in WT mice. Moreover, mutant mice, but not the WT group, revealed significant correlations between *iNos* gene expression and manifestations of behavioral impulsivity and invigoration in the open field. These findings suggest the involvement of iNOS and nitric oxide (NO) in the dysregulation of lipid metabolism and emotional behavior under conditions of genetic SERT deficiency.

Prievious research has demonstrated that brain NOS activity was inhibited by the administration of leptin, which was accompanied by increased 5-HT metabolism [73]. In the present study, the gene expression of LR isoforms A and B was lower in naive mutants compared to WT mice; WD exposure resulted in a decrease in their expression that reached statistical significance in Sert$^{-/-}$ mice but not in WT mice. A statistically significant correlation was observed between *Lr-B* gene expression and indicators of anxiety-like behavior. A negative correlation between *Lr-B* gene expression and the lipid inclusion area in Sert$^{-/-}$ mice but not in wild-type mice was shown. In the latter group, a negative correlation was identified between hepatic gene expression of *Lr-A* and the lipid inclusion area, which was not observed in the Sert$^{-/-}$ mutants. LR is implicated in adipose tissue accumulation and obesity. The reduction in gene expression of LR isoforms in both naive and WD-fed mutants may be interpreted as a regulatory adaptation to elevated leptin concentrations in these groups

[14,16]. Thus, our study demonstrates an association between LR expression and anxiety-related behavior and suggests the distinct roles of LR isoforms in behavioral and lipid metabolism in the liver of Sert$^{-/-}$ versus WT mice.

We also found genotype differences in hepatic gene expression of IR isoforms. Specifically, a downregulation of hepatic IR in non-manipulated Sert$^{-/-}$ mice was demonstrated, which is consistent with previous findings of elevated levels of phosphorylated IR substrate 1 (IRS1) and IRS2, and increased insulin levels in the liver and blood of these mice [16], as decreased *Ir* gene expression may represent a compensatory response to these increases. The two genotypes exhibited opposing effects of WD-challenge on the gene expression of IR isoforms A and B. In comparison with their respective naive counterparts, the WD-fed mutants exhibited elevated *Ir* expression, whereas the WT group demonstrated reduced gene expression of *Ir-A* and *Ir-B*. In Sert$^{-/-}$ mice, hepatic gene expression of *Ir-B* was strongly correlated with the lipid inclusion area, a phenomenon not observed in the WT group. Conversely, the latter displayed a significant negative correlation between the lipid inclusion area and hepatic gene expression of *Ir-A*, which was not revealed in Sert$^{-/-}$ mutants. Hence, in mutants, but not in WT mice, the gene expression of *Ir-A* and *Ir-B* correlated with indicators of impulsivity and anxiety. The distance to the walls correlated with *Ir-B* expression exclusively in WT mice. Sert$^{-/-}$ mice demonstrated aberrant *Ir* gene expression in the liver; these alterations displayed distinct corellations with emotionality and lipid steatosis in comparison with WT mice.

In the two genotypes, we found reciprocal differences in the expression of signaling factors and distinct correlations with behavioral characteristics of mice. The expression of all signaling factors was significantly lower in the liver of the WT/WD group and remained unaltered in Sert$^{-/-}$/WD fed mice, with the exception of the downregulated gene encoding *Cd36*. CD36 is a critical fatty acid sensor and regulator of lipid metabolism that exhibits increased expression in the liver under WD feeding regimen; it governs the uptake of long-chain fatty acids, driving hepatosteatosis [74,75]. Our findings indicated that while the gene expression of *Acsl1* significantly correlated with parameters of locomotion and impulsivity in Sert$^{-/-}$ mice, no such correlations were observed in the WT group. The latter group, however, demonstrated a strong correlation between gene expression of *Acsl1* and indicators of anxiety, which was not found in Sert$^{-/-}$ mice. Similarly, the gene expression of *Cyp4a14*, which is upregulated in the livers of obese mice fed a high-fat diet [76], exhibited a significant correlation with parameters of grooming in WT animals, but not in the mutants. This suggests its interconnection with a parameter of fear, specifically the time spent in normal posture. Notably, the gene expression of the metabolic regulators *Pten* and *Enpp1* significantly correlated with the area of lipid inclusions in the liver and the duration of freezing exclusively in the WT group, but not in Sert$^{-/-}$ mice. This observation suggests altered functions of these molecules under conditions of genetic SERT deficit. Other studies have previously demonstrated altered *Pten* expression in SERT-deficient mice [16,77]. Overexpression of *Enpp1* has previously been associated with exposure to a high-fat diet and insulin resistance [46,77]. Furthermore, inhibition of ecto-nucleotide pyrophosphatase/phosphodiesterase (ENPP1) resulted in decreased secretion and synthesis of 5-HT [47].

### *4.3 Potential Mechanisms Linking Hepatic Responses to WD and Behavior in Sert$^{-/-}$ Mice*

Notably, our investigation of Sert$^{-/-}$ and WT mice fed WD demonstrated significant correlations between altered emotionality and hepatic indicators of steatosis, as well as metabolic, inflammatory, and NO-related molecular markers. These correlations had distinct patterns between the two genotypes, suggesting the functional relevance of such relationships and the impact of genetic SERT deficit on the investigated interrelations. Several explanations for these mechanisms warrant consideration.

First, one of the primary triggering pathological features of SERT deficit is systemic endotoxinemia, resulting from the recent identification of increased intestinal permeability in Sert$^{-/-}$ mice [12]. Both highly caloric diets [78,79] and genetic SERT deficits can compromise the functionality and integrity of the intestinal barrier [11], leading to the diffusion of gut endotoxins into the bloodstream and overexpression of hepatic *Tnf* and *Il-1*$\beta$, factors that regulate anxiety-, depressive-, and aggressive-like behaviors in various mouse models [80,81]. Indeed, endotoxinemia *per se* is well documented to induce 'sterile inflammation' and the associated condition known as sickness behavior—a state characterized by depressive-like behavioral alterations and increased anxiety [82–84]. It is noteworthy that *ss*-female careers exhibit elevated basal and stress-induced levels of the pro-inflammatory cytokine IL-6 [85]. 'Sterile inflammation' can inhibit IR signaling, resulting in peripheral and central insulin resistance [86], a factor associated with anxiety and depression [87,88]. Second, hepatic steatosis can induce nitrosative stress in the liver, which is associated with neuropsychiatric syndromes [89,90]. Finally, a recent study has demonstrated abnormalities in gut microbiota that are involved in the regulation of serotonergic and lipid metabolism in aged female Sert$^{-/-}$ mice exposed to WD [42]. These abnormalities may serve as an additional factor contributing to lipid dysregulation and endotoxinemia, which subsequently impact various processes in the CNS. This explanation can be supported by previously reported correlation between microbiota changes and brain expression of *Tlr4* and *Ppargc1b*, which are markers of 'sterile inflammation' and mitochondrial functions, respectively.

## 5. Conclusions

Naive or WD-challenged aged female Sert$^{-/-}$ mice exhibited hepatic alterations in the expression of genes encoding *Ir*, *Lr*, and associated signaling molecules, as well as the gene expression of pro-inflammatory cytokines *Il-1β*, *Tnf*, and *Arg2* and *iNos*, indicators of nitrosative stress. Notably, these measures of hepatic function demonstrated significant correlation with histological parameters of liver steatosis and behavioral measures of emotionality. In numerous instances, the two genotypes manifested inverse correlations between these parameters. To summarize, our study first demonstrated a relationship between hepatic regulation of metabolism, inflammation, nitrosative stress, and emotionality in WD-challenged mice. Second, our findings suggest that genetic SERT deficiency, alone or in combination with WD challenge, alters hepatic function regulation, which may contribute to aberrant behaviors and metabolic abnormalities in Sert$^{-/-}$ mutants.

Previous research has proposed interconnected mechanisms involving insulin resistance, sterile inflammation, and other factors in the regulation of brain function under various conditions, including genetic 5-HT dysregulation [52,53]. Notably, the *ss*-polymorphism of the *SLC6A4* gene is widespread in the population, affecting 20–40% depending on ethnicity [8,91]. Given the global rise in WD-like diet consumption and the aging population, studying these mechanisms holds significant medicinal and social relevance [30]. Understanding these processes could open avenues for pharmacological targeting of hepatic mechanisms to treat both metabolic and neuropsychiatric disorders linked to obesity and reduced SERT function.

## Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Author Contributions

Conceptualization, TS, SM, BS, and RC; formal analysis, AG, ES, KS, AB, KZ, KC, KL, AD; resources, AG, BS, RC and SM; data curation, AG, KC, AB, TS, KL; writing—original draft preparation, AG, KZ, ES, AD, KL, AB, RC; writing—review and editing, RC, SM, KC, TS and AD; supervision, SM, TS; project administration, RC; funding acquisition, BS, SM, TS. All authors contributed to editorial changes in the manuscript. All authors have read and agreed to the published version of the manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

## Ethics Approval and Consent to Participate

All experimental procedures were performed in accordance with ARRIVE guidelines, the European Communities Council Directive for the Care and Use of Laboratory Animals (2010/63/EU) and approved by the local ethics committee of C. Bernard University (CBU 08RC2017). The Concent to participate is not applicable to this work a no human studies were carried out.


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## Conflict of Interest

The authors declare no conflict of interest.

## Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/FBL26778.

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