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Exposure to chronic early-life stress lastingly alters the adipose tissue, the leptin system and changes the vulnerability to western-style diet later in life in mice

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ABSTRACT

Early-life stress (ES) increases the vulnerability to develop psychopathologies and cognitive decline in adulthood. Interestingly, this is often comorbid with metabolic disorders, such as obesity. However, it is unclear whether ES leads to lasting metabolic changes and to what extent this is associated with the ES-induced cognitive impairments.

Here, we used an established chronic ES mouse model (from postnatal day (P) 2 to P9) to investigate the short- and long-term effects of ES exposure on parameters of the adipose tissue and the leptin system (i.e. circulating levels and gene expression of leptin and its receptor) in both sexes. Immediately following ES, the offspring exhibited reductions in white adipose tissue (WAT) mass, plasma leptin levels and in *leptin* mRNA expression in WAT. Furthermore, ES exposure led to increased brown adipose tissue and browning of WAT, which was evident by a drastic increase in *uncoupling protein 1* mRNA expression in the inguinal WAT at P9. Notably, the ES-induced reductions in WAT mass, plasma leptin and *leptin* expression in WAT were sustained into adulthood and were accompanied by changes in body fat distribution, such as a higher ratio between mesenteric WAT and other WATs. Interestingly, while ES exposure increased *leptin receptor* mRNA expression in the choroid plexus, it was unaltered in the hippocampus. This suggests an adaptation to maintain central leptin homeostasis following ES exposure. In addition, chronic ES exposure resulted in the well-established cognitive impairment in object recognition performance during adulthood, which correlated positively with reductions in WAT mass observed in male, but not in female mice. Finally, to assess if ES leads to a different metabolic phenotype in a moderate obesogenic environment, we measured body fat accumulation of control and ES-exposed mice in response to a moderate western-style diet (WSD) that was provided during adulthood. ES-exposed mice subjected to WSD exhibit a higher increase in adiposity when compared to controls, suggesting that ES exposure might result in a higher vulnerability to develop obesity in a moderate obesogenic environment.

To conclude, chronic ES exposure alters parameters of the adipose tissue, leads to central adaptations in leptin regulation and results in higher fat accumulations when exposed to a WSD challenge later in life. A better understanding of these metabolic effects induced by ES might open up new avenues for therapeutic (e.g. nutritional) interventions.

1. Introduction

Exposure to early-life stress (ES) leads to cognitive impairments (Chugani et al., 2001; Neigh et al., 2009) and an increased risk to develop psychopathologies in adulthood (Heim et al., 2008; Lupien et al., 2009). This is also supported by a growing number of pre-clinical studies (Avishai-Eliner et al., 2001; Naninck et al., 2015). Interestingly, not only do these psychopathologies often show comorbidity with obesity and associated metabolic disorders (Nousen et al., 2013; Milaneschi et al., 2015), but there is also (pre-)clinical evidence for associations between ES exposure and metabolic alterations later in life (Viveros et al., 2010;

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Danese and Tan 2014; Maniam et al., 2015; Lucassen et al., 2013). However, how ES exposure affects metabolic parameters exactly has so far not been addressed, nor is it known to what extent ES-induced metabolic alterations are associated with the cognitive impairments. Importantly, the development and activity of the neuronal circuits relevant for stress responses and cognition (including the hypothalamus and hippocampus) are influenced by metabolic stimuli (Harvey et al., 2006). In this respect, an endocrine organ of particular interest is the adipose tissue.

The adipose tissue is a complex endocrine and secretory organ (Casteilla et al., 2001). Mammals possess white and brown adipose tissues (WATs and BATs) that are morphologically and functionally different. The primary role of visceral (i.e. gonadal, mesenteric, perirenal, retroperitoneal) and subcutaneous (i.e. inguinal) WATs is to store excess energy in the form of lipids. In addition, WAT is considered an endocrine organ that regulates whole-body metabolism by secreting several adipokines, such as leptin (Casteilla et al., 2001), whereas BAT is involved in thermogenesis that is largely mediated through mitochondrial uncoupling protein 1 (UCP1) (Wu et al., 2013). Interestingly, white adipocytes show a "brown-like" phenotype under for example adrenergic stress (Sidossis et al., 2015) or after cold exposure (Wu et al., 2013). Under these conditions, adipocytes increase UCP1 expression and thereby their thermogenic capacity.

Leptin, the main adipokine secreted by WAT, is mostly known for its role in the regulation of food intake and energy expenditure by the hypothalamus in adulthood (Bouret and Simerly 2006). However, there is also evidence that leptin regulates circuits involved in stress responses and cognition (Farr et al., 2014). Indeed, several (pre-)clinical studies show strong associations between dysregulated leptin levels and the development of emotional and cognitive impairments (Guo et al., 2013; Milaneschi et al., 2015). In addition, leptin receptors (lep-r) are expressed, next to the hypothalamus, also in the hippocampus (Scott et al., 2009).

Circulating leptin has a typical release pattern throughout development, which consists of a peak occurring from P4 to P16 in rodents (Ahima et al., 1998). This early leptin surge is essential for the structural formation of the hypothalamic circuitry (Bouret and Simerly 2006). Considering the importance of this leptin surge, any disturbances, for example by exposure to ES, might potentially impact the developmental trajectory of these circuitries. Interestingly, both clinical and pre-clinical evidence suggests that ES does reduce leptin levels early (Salzmann et al., 2004; Schmidt et al., 2006) and later in life (Danese and Tan 2014; Viveros et al., 2010; Llorente-Berzal et al., 2011). However, it is currently unknown if and to what extent chronic ES exposure in mice affects parameters of the adipose tissue and leptin system (i.e. circulating levels and gene expression of leptin and its receptor) throughout life.

One well-known consequence of chronic ES exposure is an elevation of circulating corticosterone (CORT) early in life (Naninck et al., 2015). Because of the strong interaction between the stress system and adipocyte metabolism (Peckett et al., 2011), a rise in CORT might be involved in the possible ES-induced alterations on adipose tissues. In fact, glucocorticoid receptors (GRs) are abundantly expressed in adipose tissues and an adipocyte-specific GR knockout in mice alters the stress responses (de Kloet et al., 2015). Also, studies using other ES models have demonstrated lasting effects on central GR expression (Vázquez et al., 1996; Ladd et al., 2004), but whether ES exposure alters GR expression in adipose tissues is unknown.

Using a well-established chronic ES mouse model (Rice et al., 2008; Naninck et al., 2015), we here investigated the short- and long-term consequences of chronic ES exposure on parameters of the adipose tissue and leptin system, including fat deposition, lipid density, plasma levels of leptin as well as *leptin, UCP1* and *GR* mRNA expression in WATs. In addition, we addressed if ES exposure alters hippocampal *leptin receptor* (*lep-r*) mRNA expression. Considering that leptin entry in the brain is largely regulated via the choroid plexus (CP) (Zlokovic et al., 2000; Li et al., 2013), we also tested if ES exposure alters the *lep-r* expression in the CP.

Moreover, we included both male and female mice, considering their differential fat distribution and function (Fuente-Martín et al., 2014) and the sex-specific differences in the vulnerability to develop cognitive and metabolic disorders, such as obesity (Naninck et al., 2015; Palmer and Clegg 2015). Finally, to test if ES-exposed animals develop a different metabolic phenotype when exposed to a moderate obesogenic environment, we provided control and ES-exposed mice with a moderate western-style diet (WSD) in adulthood and monitored the changes in later body fat accumulation.

2. Material and methods

2.1. Animals

C57Bl/6J mice were purchased from Harlan Laboratories B.V. (Venray, The Netherlands) and habituated for one week before breeding. Mating occurred by housing two females (8-weeks old) with one male (6-weeks old) for one week, followed by housing pregnant females individually in clean standard cages. We observed daily between 9 a.m. and 10 a.m. for the birth of pups. When pups were born within this timeframe, the previous day was assigned as postnatal day (P) 0. All animals were maintained under constant housing conditions (temperature $22 \pm 1\,^{\circ}C$, humidity $55 \pm 5\%$, *ad libitum* standard chow and water), with a standard 12:12 h light-dark cycle schedule (lights on at 8 a.m.).

In the short-term study, male and female offspring were sacrificed at P9 (see Section 2.5). In the long-term study, animals from control (CTL) and ES conditions were transferred to clean cages with standard nesting and bedding material at P9, weaned at P21 and group-housed based on similar sex and littermates (2–3 animals/cage) and sacrificed at P180 (see Section 2.5). Food intake of adult mice (P100) was assessed for a period of 3 weeks. In the western-style diet (WSD) study, litters were randomly allocated to CTL or ES condition, male offspring were weaned at P21 and subjected to the WSD at P42 until P98.

All experiments were carried out in accordance with the Dutch legislation and European Union directives on animal experiments, were approved by the Animal Welfare Body of the University of Amsterdam and complied with principles of good laboratory animal care.

2.2. Chronic early-life stress paradigm

The chronic ES model based on limited nesting and bedding material was used to induce chronic stress in the early period (Rice et al., 2008). At P2, litters were culled to five-six pups per dam and assigned to control or stress condition until P9. Control dams received standard amounts of sawdust bedding and nesting material (one square piece of cotton material of 5×5 cm; Technilab-BMI, Someren, The Netherlands). ES dams were placed on a fine-gauge stainless steel mesh that was positioned 1 cm above a small amount of sawdust covered cage floor and reduced amounts of nesting material (2.5×5 cm). In addition, all cages contained filter tops.

2.3. Moderate western-style diet challenge

For the WSD study, mice were fed with a moderate WSD at P42-P98 (Ssniff-Spezialdiäten GmbH, Soest, Germany). The WSD was semi-synthetic and macro- and micronutrient composition was based on the American Institute of Nutrition formulation of AIN93-M purified diets for laboratory rodents, with adjustments in

fat content and composition (20% w/w fat, 3% w/w soy oil, 17% w/w lard and 0.1% w/w cholesterol).

2.4. Behavioral assessment

Learning and memory abilities of adult mice (P150) were assessed during the active phase of the light-dark cycle using the object recognition (OR) task. All mice were habituated to a box ($23.5 \times 31 \times 27$ cm) containing bedding for 5 min on three subsequent days, followed by the training day where mice were exposed to two similar objects (9.5 cm high glass bottles) for 5 min. On the testing day (24 h later), a novel object (4.5 cm yellow Lego Duplo brick) was replaced by one of the objects and mice were allowed to explore the objects for 5 min. Boxes and objects were cleaned thoroughly with 25% ethanol. Behavior was recorded using Ethovision software (Noldus, The Netherlands) and manually scored offline using the Observer program (Noldus, The Netherlands). Mice touching the object with their nose were scored as exploration. Total exploration time was compared between the groups on the training day. On the testing day, the preference of an object was expressed as the ratio of time spent with the novel relative to the familiar object (discrimination index).

2.5. Tissue collection and dissections

In the short-term study, P9 mice were quickly removed from their cages at the start of the light phase, weighed and decapitated. Trunk blood was collected in ice-cold EDTA-coated tubes (Sarstedt, Etten-Leur, The Netherlands) and centrifuged (13000 rpm, 15 min, 4 °C). Plasma was removed and stored at −20 °C until leptin analysis according to manufacturer's instructions (Milliplex Mouse Adipokine Multiplex, Millipore, Amsterdam, The Netherlands). Both hippocampi and inguinal white adipose tissue (iWAT) from the right part of the body were rapidly removed, frozen on dry ice and stored at −80 °C for further analyses. Whole interscapular brown adipose tissue (iBAT) and iWAT from the left part of the body were dissected for weight determination and post-fixed for 48 h with 4% paraformaldehyde for histological examination (see Section 2.7).

In the long-term study, P180 mice were fasted for 4 h at the start of the dark phase (reversed light-dark cycle, lights off at 8 a.m.) to minimize variations in plasma leptin due to food intake. Vaginal smears were taken from female mice to determine the estrous cycle phase. Upon decapitation, trunk blood was obtained and processed similarly as described in pups. Additionally, hippocampi and choroid plexi from the lateral ventricles were dissected separately, frozen on dry ice and stored at −80 °C until analysis. Different fat depots were dissected and weighed: iBAT, gonadal (gWAT), mesenteric (mWAT), perirenal-retroperitoneal (pWAT) and inguinal (iWAT) adipose tissues. In addition, iBAT and samples of gWAT and iWAT from the right part of the body were post-fixated with 4% paraformaldehyde for 48 h or frozen on dry-ice and stored at −80 °C until further analysis.

2.6. Body composition

Mice were weighed and body composition was assessed using dual energy x-ray absorptiometry (DEXA; Lunar PIXImus) in the WSD study (P42 and P98) and in the long-term study (P180). The DEXA scan was calibrated on the day of use with a phantom mouse (bone mineral density 0.0664 g/cm^2 and 12.5% fat). All mice were anesthetized during the procedure (<5 min) by rapid induction with a mixture of 5% isoflurane-oxygen (rate of 2 l/min), followed by placing the mouse on the scanning bed in prone position and supplied with 2% isoflurane-oxygen (rate of 2 l/min) for maintenance during the scan. Analyses were performed using the PIXImus2 software (Lunar).

2.7. Histological examination of adipose tissues

Histological examination was carried out on fixed iBATs and iWATs of P9 and P180 mice. Paraffin embedded sections (8 μm) were mounted on glass slides (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) and stained with hematoxylin and eosin (HE). Lipid density was quantified using the ImageJ 1.45s software (U.S. National Institutes of Health, Bethesda, MD, USA).

2.8. RNA preparation and quantitative real-time PCR

Frozen brain and WATs were treated with TRIzol Reagent (Invitrogen, Breda, The Netherlands) and RNA isolated according to manufacturer's instructions. Adipose tissues were additionally treated with DNase (Roche Diagnostics, Almere, The Netherlands) and applied to RNeasy mini columns (Qiagen, Venlo, The Netherlands). RNA of two lateral CPs was isolated according to manufacturer's instructions using the Quick-RNA MicroPrep kit (Zymo Research, CA, USA). To synthesize cDNA, iScript (Invitrogen, Breda, The Netherlands) was used (250 ng for hippocampal and CP tissue, 500 ng for WAT) and carried out on ABI PRISM (Applied Biosystems, CA, USA). Quantification of gene expression in WAT (*leptin, UCP1, GR*) and in hippocampus and CP (*lep-r)* were carried out (primers from Biorad, Veenendaal, The Netherlands and Thermo Fisher Scientific, Breda, The Netherlands) using SYBR Green I detection (Invitrogen, Breda, The Netherlands) or Taqman Universal Master Mix II with UNG (Thermo Fisher Scientific, Breda, The Netherlands) (Table 1). Each reaction consisted of a 10 times diluted cDNA template using this qPCR program: 95 °C for 15 min, 40 cycles, 95 °C for 15 s, 60 °C for 20 s, 72 °C for 35 s and a melting curve after 40 cycles to confirm the amplification of a single product. Efficiency of PCR amplification for all primer pairs was included when between 95 and 105% with a regression coefficient higher than 0.99. The stability of two or more housekeeping genes (HKGs) between and within groups was verified using the gene stability value (0.5 M) and coefficients of variation (0.2 CV) in the Biogazelle qBase+ 3.0 software program (Gent, Belgium). Gene expressions were normalized to the HKGs (Table 1) and experimental groups compared to the control male group.

2.9. Statistical analysis

Statistical analyses were carried out using SPSS 22.2 (IBM Software) and graphical design using GraphPad Prism (GraphPad Software, Inc). Two-way univariate F-tests with condition (CTL vs. ES) and sex (male vs female) as fixed factors were used, with Tukey's HSD as post-hoc test and mixed ANOVA to include the random factors "litter" or "estrous cycle phase" when appropriate. Mice from at least three different litters ($n \geq 6$ per experimental group) were included in each experimental group to reduce litter effects. Furthermore, the one-sample *t*-test was used to test whether the discrimination index was above the chance level of 1.0 in the OR task. Independent *t*-tests were carried out to compare the CTL and ES groups exposed to WSD. Also, Pearson correlations and linear multiple regression analyses were performed to assess correlations between OR performances with adiposity, leptin levels or *leptin* mRNA expression in WATs. All data are presented as mean ± SEM and considered significantly different when $P < 0.05$ (two-tailed).

Table 1
Oligonucleotides used for quantitative PCR in hippocampus, choroid plexus and adipose tissues.

Primers (mouse)	Primer sequences	Tissue
Designed based on NCBI database		
–Leptin	F:5′-AGGATGACACCAAAACCCTCAT-3′ R:5′-AGTCCAAGCCAGTGACCCTCT-3′	adipose tissue
–Glucocorticoid receptor	F:5′-AGGTGCCAAGGGTCTGGAGAGG-3′ R:5′-TGGTCCCGTTGCTGTGGAGGA-3′	adipose tissue
–RPL19	F:5′-TTGCCTCTAGTGTCCTCCGC-3′ R:5′-CTTCCTGATCTGCTGACGGG-3′	adipose tissue
–RPS29	F:5′-AGTCACCCACGGAAGTTCGG-3′ R:5′-GTCCAACTTAATGAAGCCTATGTCCTT-3′	adipose tissue
–CANX	F:5′-AGAGCTCAGCCTGGATCAATTC-3′ R:5′-TTGTAGTCCTCTCCACACTTATCTGG-3′	adipose tissue
Commercial gene expression assays		
–Uncoupling protein 1	qMmuCID0005832	adipose tissue
–Leptin receptor	Mm 00440181_m1	hippocampus/choroid plexus
–RPLP0	Mm 00725448_s1	hippocampus/choroid plexus
–RPL13a	Mm 02526700_g1	hippocampus/choroid plexus
–TBP	Mm 00446973_m1	hippocampus/choroid plexus

3. Results

3.1. Adipose tissue parameters are lastingly affected by chronic early-life stress

3.1.1. Short-term effects of chronic ES exposure on body weight and adipose tissue parameters

At postnatal (P) day 9, ES-exposed offspring in both sexes exhibited significantly lower body weights when compared to controls (main effect of condition: $F_{1,93} = 90.10$, $P < 0.01$ and of sex: $F_{1,93} = 4.59$, $P = 0.04$; Fig. 1a). Associated with this reduced body weight, chronic early-life stress (ES) exposure also reduced inguinal white adipose tissue (iWAT) weight (main effect of condition: $F_{1,93} = 74.83$, $P < 0.01$; no effect of sex: $F_{1,93} = 1.10$, $P = 0.30$; Fig. 1b) and increased the interscapular brown adipose tissue (iBAT) (main effect of condition: $F_{1,92} = 78.78$, $P < 0.01$; no effect of sex: $F_{1,92} = 0.67$, $P = 0.42$; Fig. 1c). We further characterized if these weight changes were due to alterations in lipid density by a HE-staining in iBAT and iWAT, which showed that ES exposure did not affect the iBAT density (% area under the curve) in males or females (no effect of condition: $F_{1,15} = 1.18$, $P = 0.30$ or sex: $F_{1,15} = 0.27$, $P = 0.61$; data not shown). HE-staining of iWAT pointed towards a possible browning of the iWAT of ES-exposed pups, which was confirmed by a 43- and 28-fold increase in the *uncoupling protein 1* (*UCP1*) gene expression in the iWAT of ES-exposed male and female pups respectively, when compared to controls (main effect of condition: $F_{1,19} = 11.00$, $P < 0.01$; no main effect of sex: $F_{1,19} = 0.08$, $P = 0.79$; Fig. 1d).

3.1.2. Long-term effects of chronic ES exposure on body weight and adipose tissue parameters

At P180, body weight of adult ES-exposed animals of both sexes were not different when compared to controls (no effect of condition: $F_{1,56} = 3.48$, $P = 0.07$; main effect of sex: $F_{1,56} = 175.77$, $P < 0.01$; Fig. 1e). While BAT weight (no effect of condition: $F_{1,26} = 0.28$, $P = 0.60$; main effect of sex: $F_{1,26} = 48.94$, $P < 0.01$; data not shown) and density (no effect of condition: $F_{1,15} = 2.24$, $P = 0.16$ main effect of sex: $F_{1,15} = 7.70$, $P = 0.01$; data not shown) were not lastingly affected by ES exposure, the weight of the different WAT depots was persistently reduced in the ES-exposed mice of both sexes. This was evident both by assessing the total body fat mass percentage determined by the DEXA scan as well as the weight of individual fat pads. These outcomes were sex and condition specific: firstly, total body fat mass was reduced in ES-exposed males (25.4%–22.5%) and females (15.0%–12.4%) (main effect of condition: $F_{1,27} = 5.46$, $P = 0.03$ and of sex: $F_{1,27} = 61.71$, $P < 0.01$; Fig. 1f), without affecting the lean body mass (no effect of condition: $F_{1,27} = 0.06$, $P = 0.81$ main effect of sex: $F_{1,27} = 84.70$, $P < 0.01$; data not shown). Furthermore, the weights of the individual WATs were reduced by ES exposure in both male as well as female adult mice (gWAT; main effect of condition: $F_{1,56} = 20.66$, $P < 0.01$ and of sex: $F_{1,56} = 40.46$, $P < 0.01$. mWAT; main effect of condition: $F_{1,56} = 5.48$, $P = 0.02$ and of sex: $F_{1,56} = 12.92$, $P < 0.01$. pWAT; main effect of condition: $F_{1,54} = 7.84$, $P < 0.01$ and of sex: $F_{1,54} = 15.25$, $P < 0.01$. iWAT; main effect of condition: $F_{1,54} = 17.77$, $P < 0.01$ and of sex: $F_{1,54} = 6.12$, $P = 0.02$; Fig. 1g). In contrast to P9, the reduction in iWAT at P180 was not associated with browning as shown by the absence of differences in either the HE-staining or in *UCP1* expression levels (no main effect of condition: $F_{1,18} = 1.94$, $P = 0.18$ or of sex: $F_{1,18} = 0.00$, $P = 0.95$; data not shown). Interestingly, while ES exposure reduced overall body adiposity, the ratio between mWAT and all other WATs was significantly higher in both ES-exposed male and female mice when compared to controls (main effect of condition: $F_{1,53} = 4.60$, $P < 0.04$ and of sex: $F_{1,53} = 6.29$, $P < 0.02$; Fig. 1h). Also, food intake of adult mice was not different between the CTL and ES groups (no main effect of condition: $F_{1,28} = 2.24$, $P = 0.15$ or sex: $F_{1,28} = 3.23$, $P = 0.08$; interaction condition*sex: $F_{1,28} = 5.97$, $P = 0.02$, no significant post-hoc results; data not shown).

3.2. Plasma leptin levels and leptin expression in adipose tissues are persistently reduced, while glucocorticoid receptor expression was unaltered after chronic early-life stress

Chronic ES exposure affected plasma levels of leptin and leptin transcription by the adipose tissues immediately after the ES exposure (at P9) as well as in adulthood (at P180) in both sexes. At P9, plasma leptin levels were reduced in the ES-exposed offspring of both sexes as compared to controls (main effect of condition: $F_{1,53} = 40.57$, $P < 0.01$; no main effect of sex: $F_{1,53} = 0.05$, $P = 0.83$; Fig. 2a). After correcting plasma leptin levels for fat mass, plasma leptin was still reduced in the ES-exposed offspring at P9 (condition: $F_{1,21} = 5.94$, $P = 0.02$). In addition, *leptin* expression in iWAT was reduced in the ES-exposed pups (main effect of condition: $F_{1,21} = 9.54$, $P < 0.01$; no main effect of sex: $F_{1,21} = 1.45$, $P = 0.24$; Fig. 2b). Furthermore, ES exposure induced similar lasting reductions in plasma leptin levels in adult mice of both sexes (main effect of condition: $F_{1,49} = 5.97$, $P = 0.02$ and sex: $F_{1,49} = 44.77$, $P < 0.01$; Fig. 2c). In the ES-exposed adult offspring, circulating leptin was not reduced after correcting the leptin levels for fat mass (condition: $F_{1,47} = 1.24$, $P = 0.27$). Interestingly, *leptin* expression in adult mice was reduced in the gWAT of the ES-exposed male and female mice (main effect of condition: $F_{1,27} = 5.65$, $P = 0.03$ and sex: $F_{1,27} = 53.15$,

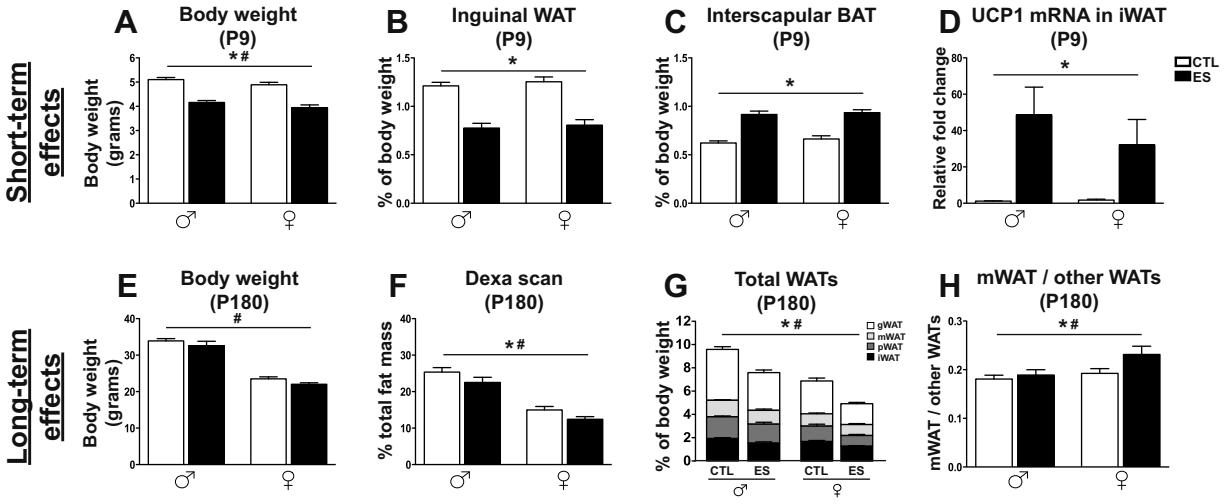

Fig. 1. Adipose tissue parameters are affected by chronic early-life stress throughout life.
(A) Early in life at P9, chronic ES exposure led to reduced body weight, (B) reduced inguinal white adipose tissue (iWAT), (C) increased interscapular brown adipose tissue (iBAT) and (D) increased *uncoupling protein 1* (*UCP1*) expression in the iWAT. (E) Later in life at P180, ES-exposed mice showed no differences in body weight when compared to controls, (F) exhibited reduced fat mass as determined by the DEXA scan and (G) by the individual fat depots, including gonadal (gWAT), mesenteric (mWAT), peri-retroperitoneal (pWAT) and inguinal white adipose tissues (iWAT). (H) ES exposure also increased the ratio between mWAT and the other WATs. Statistical analyses were performed using a two-way ANOVA: *main effect of condition, $P < 0.05$, #main effect of sex, $P < 0.05$.

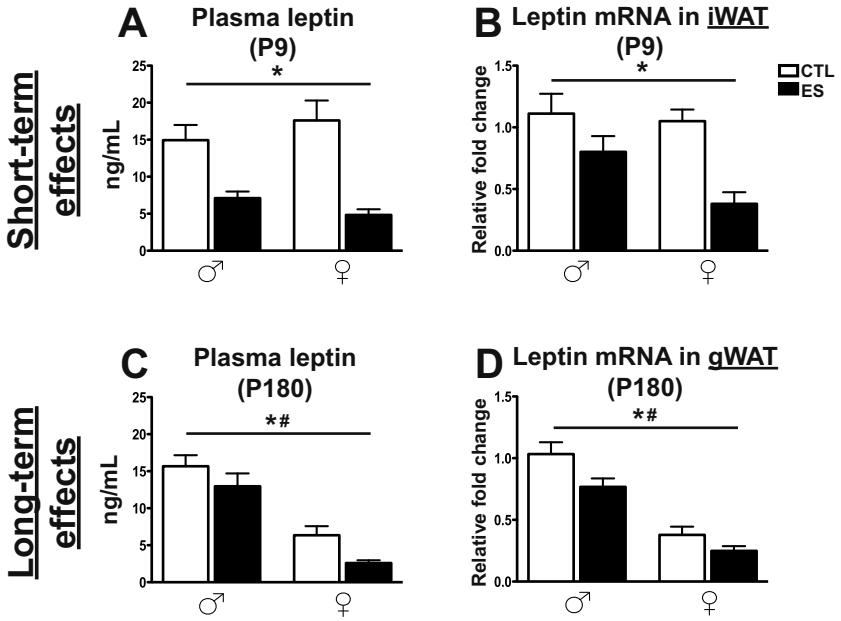

Fig. 2. Plasma leptin levels and *leptin* mRNA expression are lastingly reduced after chronic early-life stress.
(A) Early in life at P9, plasma leptin levels and (B) *leptin* mRNA expression in inguinal white adipose tissue (iWAT) were reduced after chronic ES exposure. (C) Later in life at P180, ES-exposed offspring show reduced plasma leptin levels and (D) reduced *leptin* mRNA expression in the gonadal white adipose tissue (gWAT). Statistical analyses were performed using a two-way ANOVA: *main effect of condition, $P < 0.05$, #main effect of sex, $P < 0.05$.

$P < 0.01$; Fig. 2d) and not in the iWAT (no main effect of condition: $F_{1,20} = 0.00$, $P = 0.98$; main effect of sex: $F_{1,20} = 17.34$, $P < 0.01$; data not shown). Furthermore, *glucocorticoid receptor* expression was also measured in adipose tissues, but was unaffected by ES exposure both at P9 (iWAT; no main effect of condition: $F_{1,22} = 2.38$, $P = 0.14$ or sex: $F_{1,22} = 0.09$, $P = 0.77$; data not shown) and at P180 (iWAT; no main effect of condition: $F_{1,20} = 0.48$, $P = 0.50$; main effect of sex: $F_{1,20} = 7.28$, $P = 0.01$; gWAT; no main effect of condition: $F_{1,27} = 1.11$, $P = 0.30$ or sex: $F_{1,27} = 0.17$, $P = 0.68$; data not shown).

3.3. Leptin receptor *expression is upregulated in the choroid plexus and unchanged in the hippocampus of the chronic early-life stress exposed adult mice*

Leptin receptor (*lep-r*) expression was assessed in the choroid plexus (CP; Fig. 3a) and in the hippocampus. ES exposure significantly increased *lep-r* expression in the CP by 1.5- and 1.9-fold in adult male and female mice respectively when compared to control males (main effect of condition: $F_{1,32} = 4.94$, $P = 0.03$; no main effect of sex: $F_{1,32} = 0.25$, $P = 0.62$; Fig. 3b). No differences were

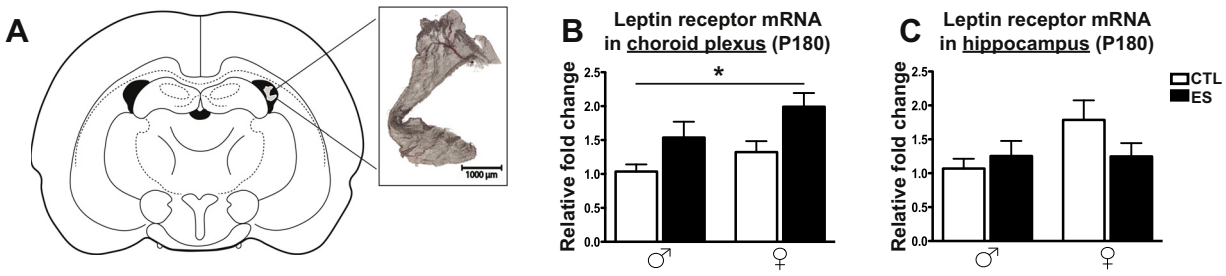

Fig. 3. *Leptin receptor* expression is upregulated in the choroid plexus and unaltered in the hippocampus of chronic early-life stress exposed adult mice.
(A) Illustration of a mouse brain depicting the location of the choroid plexus (CP) in the lateral ventricle and a photograph illustrating a dissected choroid plexus (2.5 x magnification). (B) At P180, *leptin receptor* mRNA expression was increased in the CP after ES exposure. (C) Hippocampal *leptin receptor* expression was not affected by ES exposure. Statistical analyses were performed using a two-way ANOVA: *main effect of condition, $P < 0.05$, #main effect of sex, $P < 0.05$.

found in the hippocampal *lep-r* expression between control and ES-exposed mice, neither early (no effect of condition: $F_{1,21} = 0.03$, $P = 0.88$ or sex: $F_{1,21} = 1.93$, $P = 0.18$; data not shown), nor later in life (no effect of condition: $F_{1,19} = 1.33$, $P = 0.26$ or sex: $F_{1,19} = 3.87$, $P = 0.06$; Fig. 3c).

3.4. Chronic early-life stress induced changes in adiposity are correlated with object recognition performance in male mice

Chronic ES exposure (Fig. 4a) led to the well-established cognitive impairments in the object recognition (OR) task at 5 months of age in both sexes. On the training day, there was no preference for one of the objects, neither a difference in the total exploration time between the groups (Fig. 4b). Subsequently, 24 h later on the testing day, the discrimination index was significantly above the chance level of 1.0 for control males and females (one-sample *t*-test; males: CTL $t(5) = 3.82$, $P = 0.01$; females: CTL $t(5) = 2.50$, $P < 0.05$; Fig. 4c), not for the ES-exposed males and females (one-sample *t*-test; males: ES $t(6) = 1.72$, $P = 0.14$; females: ES $t(7) = 1.93$, $P = 0.10$; Fig. 4c), and with a two-way ANOVA showing a main effect of condition ($F_{1,22} = 7.59$, $P = 0.01$) and of sex ($F_{1,22} = 12.13$, $P < 0.01$). This indicates that ES exposure impaired the OR memory and confirmed the previous reported findings by Naninck et al. (2015).

In addition, Pearson correlations were carried out to assess if parameters of the adipose tissue and leptin system are correlated with OR performances. Significant correlations were found between the amount of total WATs and OR performances in males ($R^2 = 0.43$, $P = 0.02$; Fig. 4d–e) and a trend in females ($R^2 = 0.47$, $P = 0.06$; Fig. 4d). In particular, the gWAT in male mice was significantly correlated with OR performances ($R^2 = 0.42$, $P = 0.02$; Fig. 4f). All other measured metabolic variables were not significantly correlated with OR performances in male or female mice. Further analyses using a stepwise multiple regression model demonstrated that 41% of the variances in OR performances can be explained by the gWAT in male mice ($F_{1,8} = 5.56$, $P = 0.046$; intercept: $t = 0.58$, $P = 0.58$, regression weight for gWAT: $t = 2.36$, $P = 0.046$; Fig. 4f), without significant contributions from the other metabolic variables (mWAT: $t = -1.24$, $P = 0.25$; pWAT: $t = 0.12$, $P = 0.91$; iWAT: $t = 0.01$, $P = 0.99$; plasma leptin: $t = -1.53$, $P = 0.17$).

3.5. Higher body fat accumulations after exposure to chronic early-life stress and a moderate western-style diet in adulthood

Body weight and fat composition by the DEXA scan were assessed at P42 and P98 in male mice fed with a moderate western-style diet (WSD) starting from P42 until P98 (Fig. 5a). While there were no differences in body weight between CTL and ES-exposed mice at P42 ($t(23) = 2.04$, $P = 0.053$; Fig. 5b), the ES-exposed mice demonstrated reduced total body fat mass at this age ($t(48) = 3.76$, $P < 0.01$; Fig. 5c). At P98, no differences were found in body weight ($t(23) = 0.50$, $P = 0.62$; Fig. 5d) or total fat mass ($t(23) = -0.22$, $P = 0.83$; Fig. 5e). However, mice exposed to ES and on WSD showed a higher body fat accumulation during the P42–P98 period when compared to CTL mice on WSD (Fig. 5f). In fact, when exposed to WSD, ES-exposed mice demonstrated an 1.9-fold increase in body fat at P98 as compared to P42, while CTL mice showed a lower fat mass increase of 1.6-fold ($t(23) = -2.17$, $P = 0.04$; Fig. 5 g).

4. Discussion

In the current study, we demonstrate that chronic early-life stress (ES) exposure lastingly alters various parameters of the adipose tissue and leptin system. ES exposure also leads to increased body fat accumulation in response to a moderate western-style diet (WSD) later in life. Immediately after ES exposure at P9, both male and female mice exhibited a reduced body weight accompanied by reductions in WAT mass, plasma leptin levels and *leptin* expression in iWAT as well as an increase in iBAT mass and browning of iWAT. These effects on adipose tissues were lasting, as adult ES-exposed mice of both sexes exhibited reduced weight in all the WAT pads, which was associated with reduced plasma leptin levels and *leptin* expression in gWAT. While ES resulted in reduced weights of the individual WATs, the ratio between the mesenteric WAT and other WATs was increased, pointing towards differential effects of ES on the metabolically active mesenteric fat.

Next to these peripheral alterations, ES exposure increased *lep-r* expression in the choroid plexus, which remained unaltered in the hippocampus, suggesting a compensatory adaptation to maintain central leptin homeostasis. Lastly, ES-exposed mice show higher body fat accumulations when subjected to moderate WSD as compared to non-stressed mice. Taken together, chronic ES exposure lastingly alters adipose tissue parameters, leads to central adaptations in leptin regulation and to higher fat accumulations when exposed to a moderate obesogenic environment.

4.1. Chronic ES exposure alters adipose tissue parameters and the response to a moderate western-style diet in adulthood

There is accumulating (pre-)clinical evidence that ES not only leads to persistent alterations in brain functions (Heim et al., 2008; Lupien et al., 2009; Naninck et al., 2015), but also to an increased vulnerability to develop obesity and associated metabolic disorders (Nousen et al., 2013). However, most ES-induced effects in the central nervous system are often related to emotional and cognitive dysfunction (Vázquez et al., 1996; Ladd et al., 2004). Here we provide data to support the concept that, next to the central system, ES also modulates the adipose tissue.

We found that chronic ES exposure in mice reduced body weight at P9, an effect that was no longer present at P42 and in adulthood. This is similar to the earlier described findings in rats exposed to

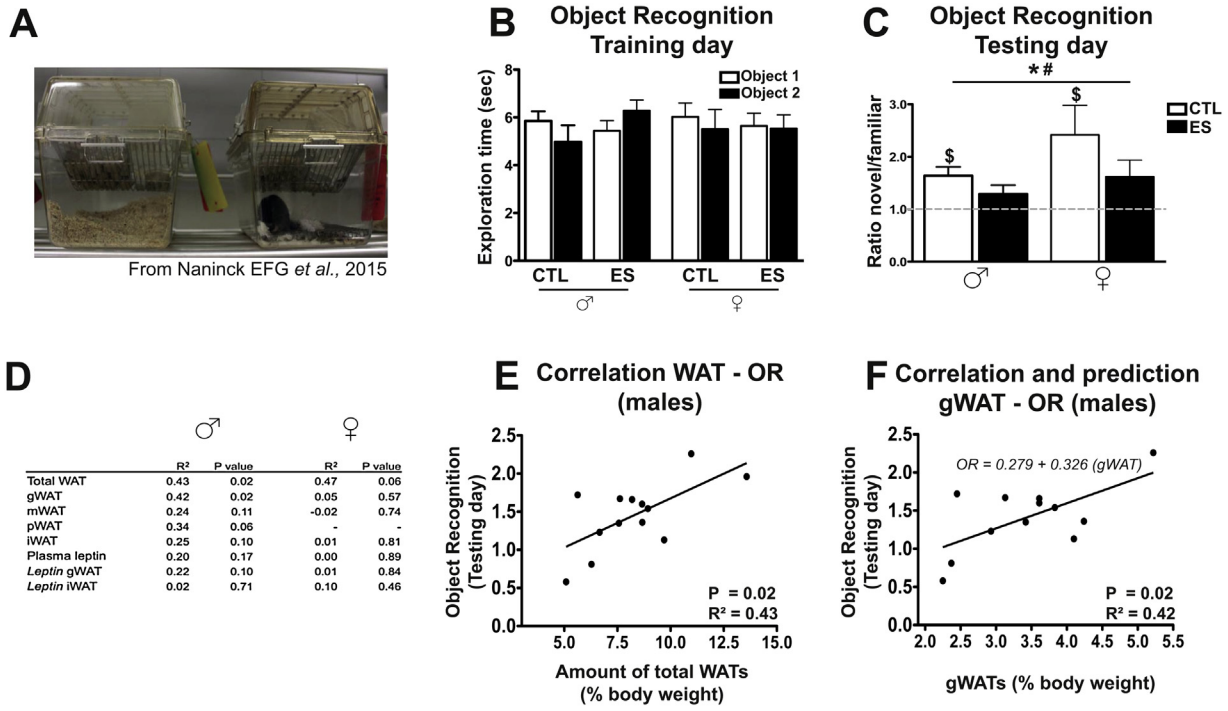

Fig. 4. Chronic early-life stress induced alterations on adiposity is correlated with object recognition performances in male mice.
(A) Photograph illustrating a control cage (left) and ES cage containing limited nesting and bedding material from P2-P9 (right). (B) On the training day, both objects were explored equally in time between the groups. (C) On the testing day, the discrimination index was significantly above chance level ($) for CTL males and females. ES-exposed mice did not perform above chance level, indicating an impaired object recognition (OR) memory. (D) Overview of correlations between parameters of the adipose tissue and leptin system and OR performances; –: did not meet the correlation assumptions. (E) Correlation plot demonstrating the association between OR and white adipose tissue (WAT). (F) Correlation and a predictive value between gonadal WAT and OR performances. Statistical analyses were performed using a two-way ANOVA: *main effect of condition, $P < 0.05$, #main effect of sex, $P < 0.05$; Pearson correlations, linear regression.

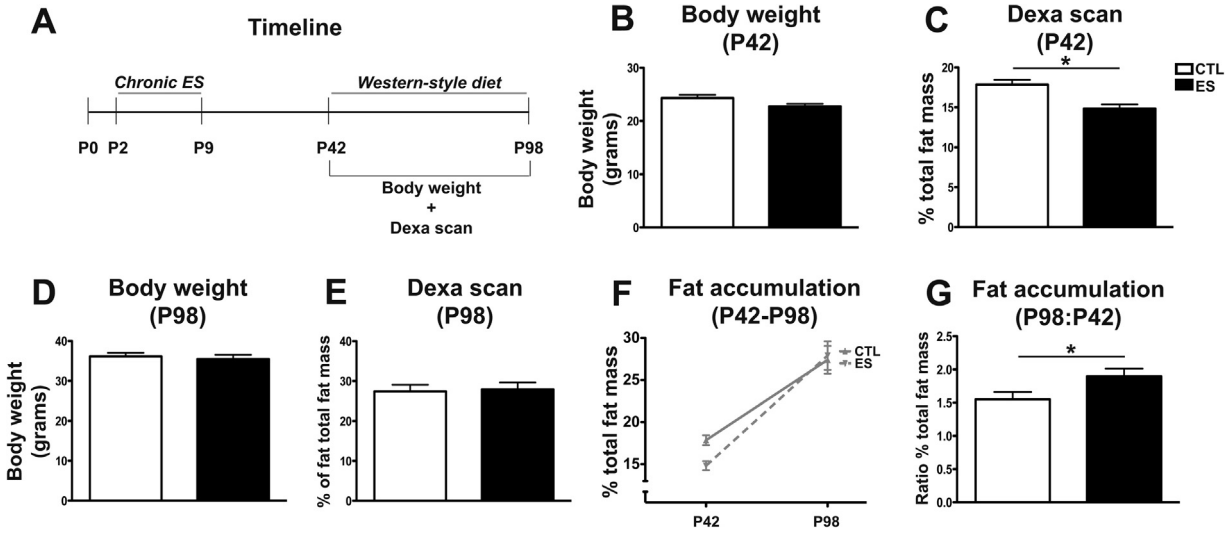

Fig. 5. Higher increase in adiposity after exposure to chronic early-life stress and a moderate western-style diet in adulthood.
(A) Overview of the western-style diet (WSD) study illustrating the period of stress and WSD exposure. (B) P42 mice showed no differences in body weight and (C) reduced body fat mass after ES exposure. (D) At P98, body weight and (E) body fat mass were not different between CTL and ES mice. (F) Increase of fat accumulation between P42 and P98 of the CTL and ES-exposed mice subjected to a WSD challenge. (G) ES-exposed mice show higher fat accumulations at P98 compared to P42. Statistical analyses were performed using an independent *t*-test, $P < 0.05$.

the same chronic ES paradigm (Maniam et al., 2015) or to maternal deprivation (Viveros et al., 2010), where a catch-up growth was present at P50. This finding, while consistent in pre-clinical literature, is in contrast with epidemiological evidence suggesting that ES (e.g. childhood maltreatment) rather leads to increases in body weight and often to obesity in adulthood (Danese and Tan 2014; Li et al., 2015).

This discrepancy led us to question how ES affects body composition in mice. Interestingly, chronic ES exposure in our mice reduced WATs in adulthood. Such reductions in fat mass appear to be species specific as they were not observed in the ES-exposed rats (Paternain et al., 2012; Maniam et al., 2015). The lasting reduction in fat mass was further associated with persistent reductions in plasma leptin at P9 and P180, which is consistent with other

pre-clinical studies (Salzmann et al., 2004; Schmidt et al., 2006; Viveros et al., 2010; Llorente-Berzal et al., 2011). Intriguingly, our ES-induced leptin reductions were also accompanied by reduced *leptin* expression in the inguinal WAT of P9 pups, whereas in adult mice, *leptin* expression was reduced in the gonadal WAT, indicating possible functional differences between specific adipose tissue depots.

Interestingly, while at first glance the reduced fat mass in the adult ES-exposed mice might appear as a leaner and 'healthier' phenotype, the higher ratio between the amount of mesenteric WAT and the other WATs might point to a less healthy outcome. In fact, the mesenteric fat depot regulates the transport of fatty acids to the liver and is therefore linked to metabolic disturbances associated with obesity (Catalano et al., 2010). Additionally, when interpreting the functional implications of the ES phenotype, one needs to consider other later life environmental factors, including diet and life-style variables known to affect adiposity. It is in fact possible that the ES-induced alterations in adipose tissues might respond differently when exposed to such elements later in life.

Indeed, we demonstrate that chronic ES exposure affects the response to a moderate WSD challenge in adulthood and leads to higher body fat accumulations. This suggests that a history of ES might result in a higher vulnerability to develop metabolic alterations, particularly when exposed to a moderate obesogenic environment. These findings are in line with other studies showing that a combination of ES exposure and palatable diets later in life (e.g. high-sugar or high-fat sucrose) increases adiposity (Paternain et al., 2012; Maniam et al., 2015). This might explain the discrepancy between the ES-induced obese phenotype in human literature and the lean phenotype we observe after ES exposure under standard dietary conditions. It is indeed likely that ES-exposed children might also live under sub-optimal conditions and could be more frequently exposed to an unhealthy nutritional regime in later life.

Next to the changes in WAT profile, chronic ES exposure increased the iBAT at P9, which returned to control levels by adulthood. Additionally, ES exposure increased *UCP1* expression in the WAT of pups at P9, indicative of WAT browning and a functional shift between different types of adipose tissues. In fact, higher *UCP1* expression, suggesting thermogenesis, and the fact that the body temperature was not affected by chronic ES exposure (unpublished observation), might suggest that ES-exposed pups were exposed to a mild cold and needed to produce more heat to maintain their body temperature. This suggest that ES might also affect the development of peripheral organs, leading to functional changes that affect the vulnerability to develop metabolic disorders later in life. Our findings support this hypothesis and suggest that chronic ES exposure in mice might program the adipose tissue for life.

4.2. What is the link between metabolic alterations and the ES-induced cognitive impairments in mice?

Leptin (increased as well as decreased levels) is proposed as a possible mediator of a common mechanism leading to metabolic disorders (e.g. obesity), psychopathologies (e.g. depression) (Nousen et al., 2013; Milaneschi et al., 2015; Ubani and Zhang 2015) and cognitive decline (Farr et al., 2008; Harvey et al., 2006; Irving and Harvey, 2014). Interestingly, next to the reduced plasma leptin levels, mice in the current study were also impaired in object recognition memory. It is interesting to consider the possible role of a lack of leptin, both during the developmental phase as well as throughout life, in modulating this process (Irving and Harvey 2014).

In the current study, we observed the well-documented sex-specific differences on fat accumulation, which was notably not differently affected by chronic ES exposure. The sex-specific vulnerability to ES-induced cognitive impairments (Naninck et al., 2015; Loi et al., 2015; Palmer and Clegg, 2015) suggests that the communication between adipocyte metabolism and brain function is differentially regulated in males versus females. Interestingly, we demonstrated significant correlations between the adipose tissues and OR performance in male, but not in female mice. This suggests that the ES-induced reduction in adipose tissues might contribute to the cognitive impairments observed in male mice only, which might underlie some of the sex-specific ES effects (Naninck et al., 2015; Loi et al., 2015). However, further studies are needed to clarify this.

The fact that no correlations were found between plasma leptin or *leptin* mRNA levels and OR performances in male or female mice can be interpreted in multiple ways. On the one hand, leptin levels in the brain could be critical modulators of cognition (Farr et al., 2014; Harvey et al., 2006) and that central levels of leptin are therefore tightly controlled and preserved despite reduced circulating leptin levels. In agreement, we did not observe differences in *lep-r* expression in the hippocampus after chronic ES exposure in P9 or P180 mice. Notably, the prefrontal cortex and amygdala are also required for OR performances, but the possible influence of leptin on the functionality of these brain regions were not considered as the presence of lep-r is less well described in these brain regions (Cohen and Stackman 2015; Patterson et al., 2011; Scott et al., 2009).

We further observed a strong upregulation of *lep-r* expression in the choroid plexus (CP). The presence of lep-r in the CP is crucial for regulating leptin entry into the brain (Zlokovic et al., 2000; Li et al., 2013). While little is known about its regulation, lep-r is influenced by the fasting status (Mitchell et al., 2009). Also, adult rats exposed to maternal separation show changes in genes specifically expressed in the CP (Kohda et al., 2006). In our study, the ES-induced upregulation of *lep-r* expression in the CP could point towards an adaptive response to the life-long reduction in peripheral leptin levels in order to maintain central leptin homeostasis. In line with this possibility, direct administration of leptin to the hippocampus modulates cognition (Farr et al., 2006), whereas peripheral leptin treatment failed to affect the HPA axis response to maternal separation (Salzmann et al., 2004; Schmidt et al., 2006). Nevertheless, peripheral leptin injections regulate adrenal sensitivity to elevations in ACTH occurring during prolonged separation periods, indicating that leptin levels might be altered peripherally but remain preserved centrally. On the other hand, it is possible that, next to leptin, other metabolic factors are responsible for the central effects on cognition and the ES-induced alterations in adipose tissue. In this respect, other adipokines, such as adiponectin, might play important roles in regulating hippocampal functioning (Diniz et al., 2012).

4.3. What mediates the chronic ES-induced alterations in adipose tissues?

The data presented in this paper suggest that chronic ES exposure lastingly alters the fat mass and their capacity to transcribe leptin. Similarly to the brain (Lupien et al., 2009), the development of adipose tissues in rodents occurs early in life during the last week of gestation and early postnatally (Lukaszewski et al., 2013). Perturbations during this sensitive period could lead to lasting alterations in adipocytes. Indeed, exposure to prenatal stress in combination with a high-fat diet in later life resulted in higher adiposity in adulthood (Tamashiro and Moran 2010). It is however unknown what mediates such alterations in adipocytes after ES, but a likely candidate in this respect is glucocorticoids. Adipocytes are rich in glucocorticoid receptors (GRs) and corticosterone (CORT) affects adipocyte function (i.e. fat mass and leptin release) (Zakrzewska et al., 1997; Masuzaki 2001). In our model, ES-exposed mice exhibit strong elevated basal CORT levels at P9 (Rice et al., 2008; Naninck

et al., 2015), which could contribute to the altered metabolic phenotype. While ES exposure lastingly alters central GR expressions in rats (Vázquez et al., 1996; Ladd et al., 2004) and mice (García-Gutiérrez et al., 2016), we found no differences on *GR* expression in WATs after chronic ES exposure at P9 and P180, suggesting that GR expression is differently regulated in the brain and adipose tissue.

In summary, chronic ES exposure persistently alters parameters of the adipose tissue and leptin system in both sexes, and leads to higher body fat accumulations when exposed to a moderate WSD in adulthood. Further understanding of these metabolic alterations and its relation to the central effects induced by chronic ES might help to develop specific therapeutic (peripheral) interventions to target vulnerable populations exposed to ES..

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