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## Ovariectomy-induced depressive-like behavior and brain glucose metabolism changes in female rats are not affected by chronic mild stress

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### ABSTRACT

The increased incidence of depression in women going through peri-menopause suggests that fluctuations in estrogen levels may increase the risk of developing depression. Nonetheless, this psychiatric disorder is likely to be multifactorial and consequently an additional trigger may be needed to induce depression in this population. Stress could be such a trigger. We therefore investigated the effect of ovarian estrogen depletion and chronic mild stress (CMS) on depressive-like behavior and brain metabolism in female rats.

Approximately 2 and 9 weeks after estrogen depletion by ovariectomy, behavioral changes were assessed in the open-field test and the forced swim test, and brain metabolism was measured with [<sup>18</sup>F]FDG PET imaging. A subset of animals was subjected to a 6-weeks CMS protocol starting 17 days after ovariectomy.

Short-term estrogen depletion had a significant effect on brain metabolism in subcortical areas, but not on behavior. Differences in depressive-like behavior were only found after prolonged estrogen depletion, leading to an increased immobility time in the forced swim test. Prolonged estrogen depletion also resulted in an increase in glucose metabolism in frontal cortical areas and hippocampus, whereas a decrease glucose metabolism was found in temporal cortical areas, hypothalamus and brainstem. Neither short-term nor prolonged estrogen depletion caused anxiety-like behavior. Changes in body weight, behavior and brain glucose metabolism were not significantly affected by CMS.

In conclusion, ovarian estrogen depletion resulted in changes in brain metabolism and depressive-like behavior, but these changes were not enhanced by CMS.

### 1. Introduction

Depression is a common mental disorder with symptoms like loss of pleasure or interest in daily life activities (anhedonia), poor concentration, decreased energy, insomnia or hypersomnia, weight loss or gain, and others (Post and Warden, 2018). These symptoms may result in poor quality of life, accompanied by a decrease in productivity and increased mortality from suicide (WHO, 2017). This neuropsychiatric disorder is currently the leading cause of disability in the world, with more than 300 million people being affected (WHO, 2017).

Epidemiological studies of depression have shown that the disease is twice as common in women as in men (Kessler et al., 1993; Weissman

et al., 1984). Recent evidence suggests that estrogens play an important role in the pathophysiology of depressive disorders in women (Schmidt et al., 2015; Slowik et al., 2017; Whooley et al., 2000). This could explain the increased risk for women to develop depression during periods of hormonal changes, such as pre-menstrual, during pregnancy and post-partum, and both peri- and post-menopausal periods (Bloch et al., 2015; Deecher et al., 2008; Georgakis et al., 2016; Gordon et al., 2015; Schmidt et al., 1998; Vivian-Taylor and Hickey, 2014).

Thus, the increased risk for women during peri-menopause to develop depression, could be explained by the decline in circulating estrogens (Grochans et al., 2018; Schmidt et al., 2015; Soares, 2017). However, since only a minority of women with ovarian estrogen

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depletion suffer from depression (Soares, 2014), it seems plausible that the incidence of depression in postmenopausal women depends not only on estrogen levels, but probably also requires an additional trigger. Stress might be such a trigger; demographic studies suggest that stressful life events during the transition to menopause could contribute to the development of depression (Cohen et al., 2006; Hankin and Abramson, 2001; Schmidt et al., 2004). Therefore, we hypothesized that the combination of stressful life events and declining estrogen levels may culminate in a higher incidence of depression.

The objective of this study was therefore to determine whether chronic mild stress (CMS) during a period of estrogen depletion affects the susceptibility of female rats to develop depressive-like behavior. Ovariectomy was performed to decrease circulating estrogen levels in female rats. Two weeks after ovariectomy, the animals were exposed to a 6-weeks CMS protocol, involving continuous exposure to a variety of mild stressors, known to induce learned helplessness, a core symptom of depression in humans (Willner, 2005). Depressive-like behavior was assessed in the open-field test and the forced swim test before and after the CMS protocol. In addition, [ $^{18}\text{F}$ ]FDG PET scans were performed as a marker of brain metabolism. Changes in brain metabolism have been related to hormonal changes and depressive-like behavior (Coyoy and Universitaria, 2016; Ernst et al., 2002; López and Tena-Sempere, 2015; Santiago et al., 2016; Silverman et al., 2011). In fact, clinical [ $^{18}\text{F}$ ]FDG PET imaging studies have shown that glucose metabolism is affected in various parts of the brain of depressive patients (Su et al., 2014).

## 2. Materials and methods

### 2.1. Animals

Female outbred Wistar rats ( $n = 32$ , 9–12 weeks old, 200–250 g) were purchased from Harlan (Horst, The Netherlands). Animals were housed individually in Macrolon cages filled with wood shavings, placed in a room with a constant temperature ( $21 \pm 2^\circ\text{C}$ ) and a fixed 12-h light-dark regime. Standard laboratory chow and water were available ad libitum. After arrival, rats were acclimatized for at least 7 days. During acclimatization and throughout the study, all rats were handled daily. All experiments were approved by the Institutional Animal Care and Use Committee of the University of Groningen, The Netherlands (study protocol: DEC 5842D).

### 2.2. Study design

The timeline of experimental procedures is depicted in Fig. 1. All rats were ovariectomized and divided into two treatment groups, placebo (VEH) and estradiol (EST), and further divided into two condition groups, animals without (CTL) and with exposure to chronic mild stress (CMS) for six weeks. This led to four different groups of 8 ovariectomized animals: placebo alone (VEH + CTL), estradiol replacement

**Table 1**

Chronic mild stress protocol. Chronic mild stress (CMS) was induced during 6 weeks, with fixed stressors every day.

Day	Stressors
Sunday	20:00 h: Paired housing for 14 h
Monday	10:00 h: Tilting of the cages ( $45^\circ$ ) for 10 h 20:00 h: Soiled cage (250 ml of water was poured into the sawdust bedding) for 14 h
Tuesday	10:00 h: Cage cleaning, followed by water deprivation for 10 h 20:00 h: Paired housing for 14 h
Wednesday	10:00 h: Stroboscopic illumination in darkness for 10 h 20:00 h: Food deprivation for 14 h
Thursday	10:00 h: Tilting of the cages ( $45^\circ$ ) for 10 h 20:00 h: Cages were put back in straight position. Animals were allowed a period without exposure to a stressor
Friday	10:00 h: Stroboscopic illumination in darkness for 10 h 20:00 h: Soiled cage (250 ml of water was poured into the sawdust bedding) for 14 h
Saturday	10:00 h: Cage cleaning followed by no stress 20:00 h: Food and water deprivation for 14 h

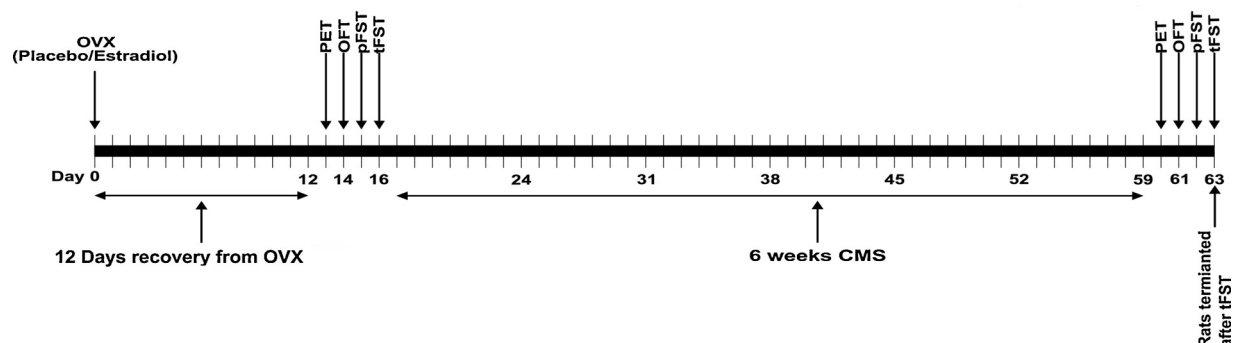
(EST + CTL), placebo in combination with stress (VEH + CMS), and estradiol replacement in combination with stress (EST + CMS).

On day 0 the rats were ovariectomized and either a placebo or an estradiol releasing pellet was implanted. Rats were allowed to recover from the surgery for 12 days. On day 13, a [ $^{18}\text{F}$ ]FDG PET scan was performed, followed by an open field test (OFT) on day 14 and the forced swim test (FST) on days 15 (pretest) and 16 (test). Between day 17 and day 59 (6 weeks), half of the rats were exposed to mild stressors according to the CMS protocol (Table 1). Control animals were not exposed to these stressors. On day 60, all rats were subjected to another [ $^{18}\text{F}$ ]FDG PET scan, followed by an OFT on day 61, and FST on day 62 (pretest) and 63 (test). Immediately after the last FST test, all animals were sacrificed and the brain, uterus and adrenal glands were dissected.

### 2.3. Ovariectomy and estradiol replacement

Bilateral ovariectomy was performed under isoflurane anesthesia as previously described (Khayum et al., 2014). Both sides of the abdomen were shaved and sterilized with 70 % ethanol. A small incision was made in the skin and muscle layer of the abdomen to expose the ovaries. The blood vessels that supply the ovaries were ligated with silk sutures and the ovaries with the associated fat pads and a part of the uterus were removed. The muscle layer and the skin were then closed with chromic sutures.

Immediately after ovariectomy, a placebo pellet or an estradiol-releasing pellet (NC-111 Placebo, and NE-121  $17\beta$ -estradiol 2.25 mg/pellet, 90 day release, Innovative research of America, Florida, USA) was subcutaneously implanted in the neck of the rat. Estradiol pellets released 25  $\mu\text{g}$  of estradiol per day with zero order kinetics, generating



**Fig. 1. Study design.** On day 0 rats were ovariectomized (OVX) and implanted with either an estradiol or vehicle releasing pellet. After 12 days of recovery, rats were exposed to a baseline PET scan and behavioral tests (OFT: open field test, pFST: pre-forced swim test, tFST: test forced swim test). On day 17, animals were exposed to chronic mild stress (CMS) for 6 weeks. After the CMS protocol, the PET scan, OFT, pFST and tFST were repeated.

physiological levels of estradiol in plasma. After surgery, the rats were subcutaneously injected with finadyne (2.5 mg/kg Flunixin, Schering-Plough N.V./S.A., Belgium) for pain relief. Administration of finadyne was repeated 24 h after surgery. Rats were weighed weekly to monitor body weight changes.

#### 2.4. Chronic mild stress

The CMS protocol started on day 17 and consisted of 6 weekly cycles of exposure to different stressors (Table 1), as described elsewhere (Dalla et al., 2005; Dioli et al., 2019; Grippo et al., 2005; Pitychoutis et al., 2012). The stressors were as follows: paired housing (each rat 6x acted as a resident and 6x as an intruder), tilting of cages to 45°, soiled cage, stroboscopic illumination (1500 W, 2 flashes per second), and food and water deprivation. Control rats were housed in a separate room, and were not exposed to these stressors.

#### 2.5. Open field test

The open field test was performed twice on days 14 and 61. Animals were placed in the center of a circular black arena (diameter 80 cm, height 40 cm) and were allowed to explore for 5 min. During this period, behavior was videotaped for subsequent behavioral analysis. After each open field experiment, the arena was cleaned with water and 70 % alcohol. Videos were analyzed using Ethovision XT 8 (Noldus Information Technology, Wageningen, The Netherlands). The outcome parameters were the total distance traveled by the animal (locomotion) and the time spent in a central circular area with a 40 cm diameter (anxiety).

#### 2.6. Forced swim test

On day 15 and 62, animals were subjected to a pretest FST. Rats were individually placed in a cylindrical water tank (diameter 20 cm; height 40 cm) for 15 min. The water level in the tank was set at 30 cm to ensure the rats could swim or float without touching the bottom of the tank with their hind limbs. Water temperature was maintained at  $21 \pm 1$  °C. After 15 min, animals were taken out of the cylinder. On day 16 and 63, rats were subjected to the test FST by placing the animals in the cylinder filled with water, under the same conditions as in the pretest, but only for 5 min. After every exposure to the cylinder, rats were dried with paper towels and returned to their home cages. After every session, water from the tank was discarded; the tank was washed and filled with fresh water for the next test. The entire pretest and test FST sessions were recorded on video for subsequent analysis using Ethovision XT 8 software (Noldus Information Technology, Wageningen, The Netherlands).

Floating and high locomotion time were used as outcome parameters of helplessness. Floating was defined as less than 2 % change in body surface area, while highly locomotion was defined as more than 12 % change in body surface area. Parameters were measured in a 5 min exposure to the FST cylinder.

#### 2.7. [<sup>18</sup>F]FDG PET imaging

On days 13 and 60, animals were subjected to an [<sup>18</sup>F]FDG PET scan. Rats were placed in cages with a temperature of 30 °C for environmental adaptation and minimization of [<sup>18</sup>F]FDG uptake in brown fat. After 30 min of adaptation, rats were anesthetized with isoflurane (5 % induction and 2 % maintenance, in medical air) and injected with [<sup>18</sup>F]FDG via the tail vein ( $14.5 \pm 2.5$  MBq). Rats were allowed to recover from anesthesia in their cages. Forty min after injection, the rats were again anesthetized with isoflurane and placed in a small animal PET camera (Focus 220, Siemens Medical Solutions, USA, Inc). Rats were placed in a transaxial position with their heads inside the field of view. 45 min after [<sup>18</sup>F]FDG injection, a 30 min static PET scan

was acquired. During the PET scan, rats were kept under isoflurane anesthesia (2 % in medical air) and body temperature was maintained by heating pads. Eye salve was used to prevent dehydration of the eyes. After completion of the emission scan, a transmission scan with a <sup>57</sup>Co point source was acquired for 515 s to allow correction of attenuation and scatter by tissue. List mode emission data was iteratively reconstructed into a single frame image of 30 min (OSEM2D, 4 iterations, and 16 subsets). PET data were normalized and corrected for attenuation, scatter, random coincidences and radioactive decay.

#### 2.8. PET analysis

To determine global and region-of-interest differences in [<sup>18</sup>F]FDG brain uptake, volumes of interest (VOI) were drawn on a [<sup>18</sup>F]FDG brain template that was co-registered with the PET images. Region of interest (ROI) analysis was performed with a brain template including the following areas of interest: amygdala, bed nucleus stria terminalis, brainstem, cerebellum, frontal cortex, hippocampus, hypothalamus, insular cortex, midbrain, occipital cortex, olfactory cortex, parietal cortex, striatum, temporal cortex, thalamus and whole brain. The VOIs from the template were copied to the individual PET images to determine the accumulation of radioactivity in the whole brain and individual brain regions (in Bq/cm<sup>3</sup>). [<sup>18</sup>F]FDG uptake was corrected for body weight and injected dose and expressed as the standardized uptake value (SUV). The SUV was defined as: [tissue activity concentration (Bq/cm<sup>3</sup>)]/[injected dose (Bq)/body weight (g)]. It was assumed that 1 cm<sup>3</sup> of brain tissue equals 1 g.

#### 2.9. Statistical analysis

All data is expressed as mean  $\pm$  standard error of the mean (SEM). All statistical analyses were performed using IBM SPSS statistics 22 for Windows. Body weight changes, behavioral changes (OFT and FST), and [<sup>18</sup>F]FDG uptake (expressed as SUV) were analyzed using a General Estimated Equation model (GEE). Between-group and within-group differences were assessed using the factors: treatment (estradiol vs. placebo), condition (chronic mild stress vs. control) and time. Additionally, to determine the interaction between estrogen depletion (EST) and chronic mild stress (CMS), an interaction analysis was performed with the model using the data of the scans performed after the CMS protocol.

For analysis of adrenal gland and uterus weight changes, data was normalized to body weight, and analyzed using one-way ANOVA and Bonferroni as post-hoc test. For all statistical analyses, significance was assumed when the probability (p) was < 0.05. Moreover, effect sizes were estimated for all PET analyses, using the *d* index, which is defined as:

$$\text{Cohen's } d = (M_2 - M_1) / SD_{\text{pooled}}$$

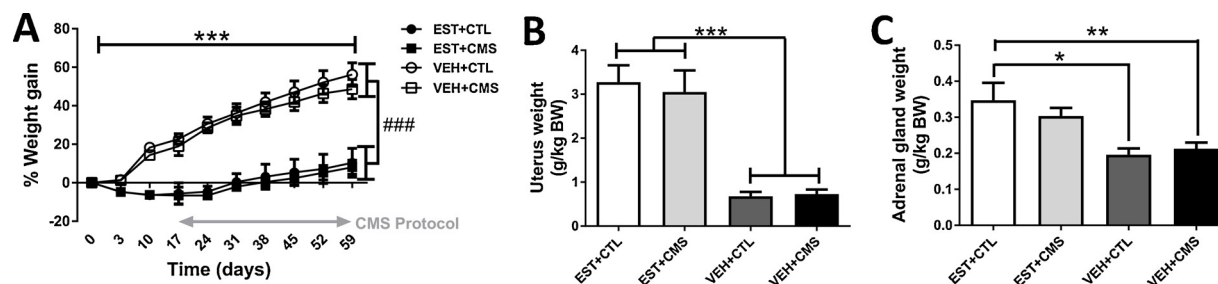
where

$$SD_{\text{pooled}} = \sqrt{((SD_1^2 + SD_2^2) / 2)}$$

### 3. Results

#### 3.1. Physiological parameters

Body weight was measured weekly in order to assess the effect of estrogen depletion and CMS. Within-group differences in body weight gain over time were observed in all experimental groups (Fig. 2A;  $p < 0.001$ ), although at different rates. Significant differences between groups were first found on day 10 (i.e. before the CMS protocol; Fig. 2A), with animals receiving estrogen replacement (EST + CTL  $-4.7 \pm 1.2$  g; EST + CMS  $-4.8 \pm 0.8$  g) showing significantly ( $p < 0.001$ ) less body weight gain than estrogen-depleted animals (VEH + CTL  $1.5 \pm 0.5$  g; VEH + CMS  $1.2 \pm 0.7$ ). At the end of the



**Fig. 2. Physiological parameters.** A. Body weight changes during the experiment ( $n = 8$  per group). B. Normalized changes in the uterus weight at the end of the experiment (EST + CTL  $n = 8$ ; EST + CMS  $n = 6$ ; VEH + CTL  $n = 8$ ; VEH + CMS  $n = 6$ ). C. Normalized adrenal gland weight changes after all experimental procedures (EST + CTL  $n = 7$ ; EST + CMS  $n = 8$ ; VEH + CTL  $n = 8$ ; VEH + CMS  $n = 8$ ). Data is presented as mean  $\pm$  SEM. Statistically significant differences between groups are indicated as: \* $p < 0.05$ ; \*\* $p < 0.01$  and \*\*\* $p < 0.001$ . Differences within groups are indicated as ###  $p < 0.001$ .

experiment, the increase in body weight in animals with estradiol replacement, as compared to estrogen-depleted animals, was even more pronounced (EST + CTL  $10.4 \pm 7.0$  g vs. VEH + CTL  $56.2 \pm 5.7$  g,  $p < 0.001$ ; EST + CMS  $8.1 \pm 3.4$  g vs. VEH + CMS  $48.6 \pm 4.7$  g;  $p < 0.001$ ). No effect of stress alone was found at any time point, when comparing the VEH + CTL group vs. the VEH + CMS group or the EST + CTL group vs. the EST + CMS group.

The weight of the uterus, normalized for body weight, was measured at the end of the experiment (day 63), as an indicator of changes in circulating estrogen levels (Fig. 2B). Estrogen depletion had a significant effect on uterus weight, resulting in a significantly ( $p < 0.001$ ) lower uterus weight in the estrogen-depleted animals (VEH + CTL  $0.7 \pm 0.10$  g/kg; VEH + CMS  $0.7 \pm 0.10$  g/kg) than in the estradiol-treated groups (EST + CTL  $3.3 \pm 0.38$  g/kg; EST + CMS  $3.1 \pm 0.48$  g/kg). Uterus weight was not affected by CMS.

The adrenal gland weight, normalized to body weight, was measured to assess the effect of chronic activation of the hypothalamus-pituitary-adrenal (HPA) axis by stress (Fig. 2C). Estrogen depletion induced shrinkage of the adrenal gland, resulting in a significantly lower adrenal gland weight in estrogen-depleted animals than in the estradiol replacement group that was not exposed to CMS, (EST + CTL  $0.35 \pm 0.05$  g/kg vs. VEH + CTL  $0.20 \pm 0.02$  g/kg, or VEH + CMS  $0.21 \pm 0.02$  g/kg;  $F = 1.09$ ,  $p < 0.05$ ,  $p < 0.01$ , respectively). The adrenal gland weight of the EST + CMS group ( $0.31 \pm 0.02$  g/100 g) was not significantly different from any of the other groups. No effect of CMS on adrenal gland weight was observed.

These results show that estrogen depletion increased body weight and reduced uterus and adrenal gland weight, whereas CMS did not have any effect on these parameters.

## 3.2. Behavioral tests

### 3.2.1. Open field test

The OFT was used to determine motor activity and anxiety-like behavior. Time spent in the center of the arena was used as a measure of anxiety-like behavior (Fig. 3A–B). No significant effects of estrogen-depletion on the total time spent in the center was found on day 14 (EST  $16.6 \pm 8.7$  s; VEH  $24.9 \pm 5.9$  s). On day 61, no significant effects of estrogen-depletion or CMS on anxiety-like behavior were observed either (EST + CTL  $16.1 \pm 4.3$  s; EST + CMS  $26.0 \pm 9.9$  s; VEH + CTL  $20.7 \pm 4.7$  s; VEH + CMS  $18.1 \pm 7.1$  s). There were also no significant changes between day 14 and 61 in any group.

On day 14, there was no effect of estrogen-depletion on locomotion, measured as total distance moved (EST + CTL  $15.5 \pm 2.2$  m; VEH + CTL  $13.3 \pm 1.0$  m). No significant differences in distance moved between groups were found on day 61 either (EST + CTL  $12.6 \pm 2.1$  m; EST + CMS  $15.0 \pm 3.7$  cm; VEH + CTL  $14.0 \pm 1.7$  m; VEH + CMS  $11.6 \pm 2.3$  m). No effects of time or treatment on motor activity were found in a within-group comparison analysis (Fig. 3C–D).

So, neither estrogen depletion nor CMS affected motor activity or

anxiety-like behavior in the OFT.

### 3.2.2. Forced swim test

Floating time was used as a parameter of learned helplessness in the FST (Fig. 3E–F). No effect of estrogen depletion on floating time was found on day 16 (EST  $41.8 \pm 5.6$  s; VEH  $29.1 \pm 8.1$  s). Nonetheless, an increase in floating time due to estrogen depletion was found in stress naïve animals on day 63 (EST + CTL  $26.8 \pm 10.6$  s; VEH + CTL  $99.2 \pm 36.5$  s;  $p < 0.05$ ). In animals that underwent the CMS protocol, a similar trend was observed, although the effect of estrogen depletion in these animals was not statistically significant yet (EST + CMS  $25.3 \pm 9.8$  s; VEH + CMS =  $63.5 \pm 23.8$  s). No effect of CMS on floating time was observed.

High locomotion was measured as a state of motivation of the animal to escape from the cylinder (Fig. 3G–H). Short-term effects of estrogen depletion on high locomotion could not be detected on day 16, but significant long term effects of estrogen depletion were found on day 63 (EST + CTL  $66.1 \pm 15.9$  s; EST + CMS  $68.7 \pm 9.5$  s; VEH + CTL  $21.6 \pm 5.4$  s; VEH + CMS  $34.9 \pm 7.0$  s;  $p < 0.01$ ). No significant effect of stress alone was observed.

Thus, estrogen depletion seems to increase learned helplessness and decrease motivation in the FST, whereas CMS did not have any effect.

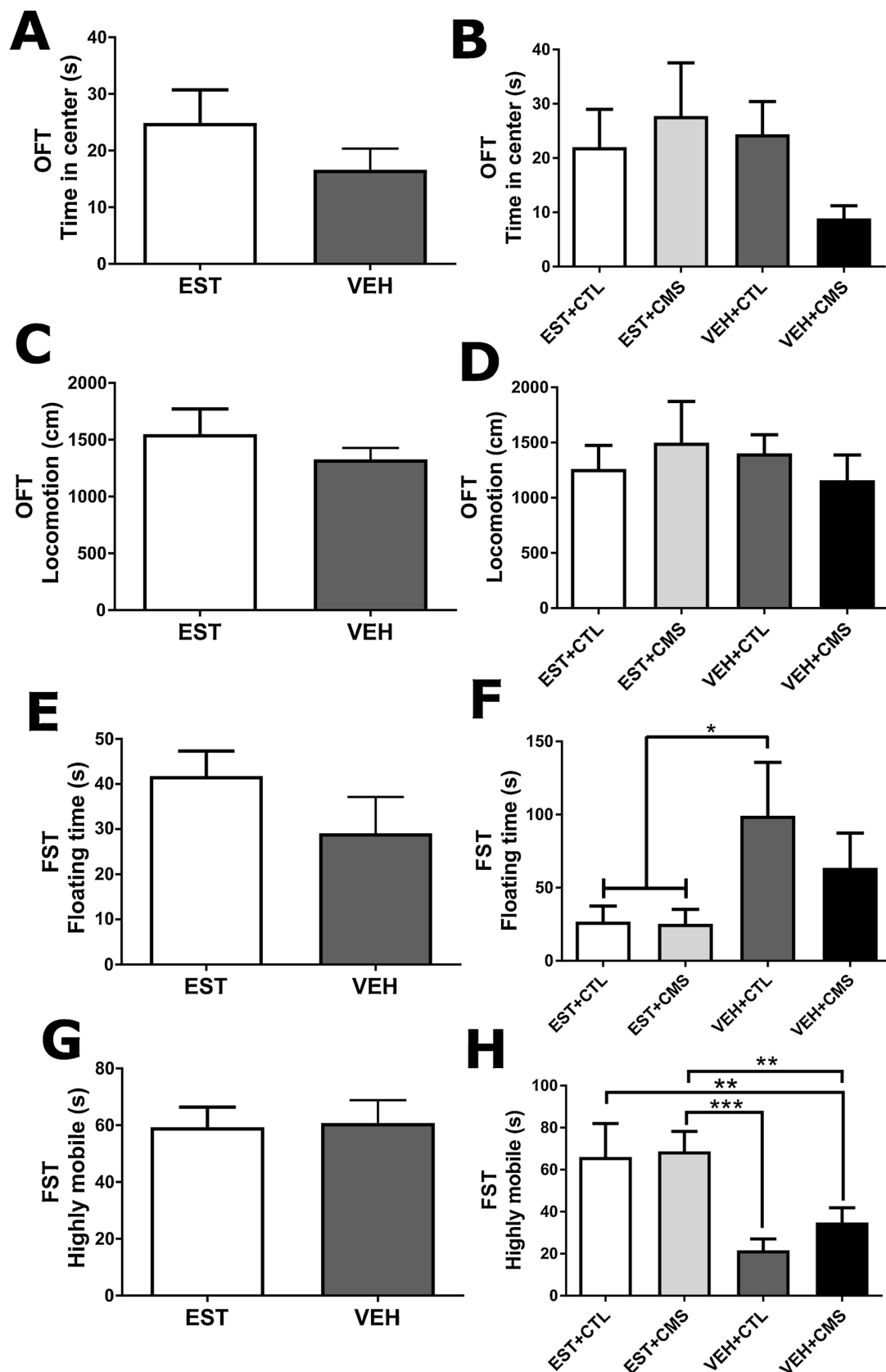
## 3.3. [ $^{18}$ F]FDG PET

### 3.3.1. Regional glucose metabolism changes 13 days after OVX

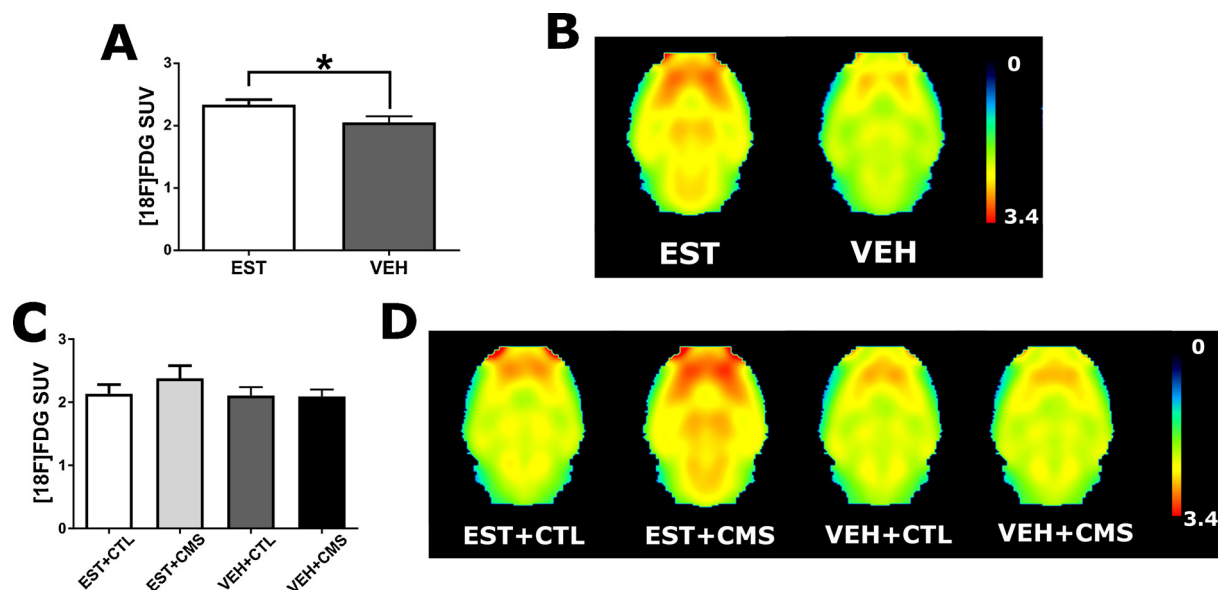
Whole brain [ $^{18}$ F]FDG uptake, as a surrogate biomarker of cerebral glucose metabolism, is shown in Fig. 4A–B. A statistically significant increase in whole brain [ $^{18}$ F]FDG uptake between estradiol- and placebo-treated rats was found on day 13 ( $p < 0.05$ ; EST SUV =  $2.3 \pm 0.1$ ; VEH SUV =  $2.05 \pm 0.1$ ). ROI analysis showed that after 13 days of estrogen depletion, placebo-treated rats presented a decrease in glucose metabolism in several areas, including midbrain, hippocampus, thalamus, cerebellum, brainstem, frontal cortex, striatum, insular cortex and bed nucleus stria terminalis, when compared to estradiol-treated animals (Table 2,  $p < 0.05$ ,  $p < 0.001$ ).

### 3.3.2. Regional glucose metabolism changes after CMS

Changes in brain glucose metabolism on day 60 are presented in Fig. 4C–D. No statistically significant effects of treatment (placebo or estradiol), condition (control or CMS) or interaction between treatment and condition in whole brain [ $^{18}$ F]FDG uptake were found (EST + CTL SUV =  $2.1 \pm 0.1$ ; EST + CMS SUV =  $2.4 \pm 0.2$ ; VEH + CTL SUV =  $2.1 \pm 0.1$ ; VEH + CMS SUV =  $2.1 \pm 0.1$ ). On day 60, estrogen depletion alone induced significant differences in brain metabolism (Table 3). When comparing groups without CMS (EST + CTL vs VEH + CTL), significantly higher [ $^{18}$ F]FDG uptake was found in the insular cortex of rats with estradiol treatment, when compared to vehicle control animals ( $p < 0.05$ ). When comparing groups with CMS (EST + CMS vs VEH + CMS), a higher uptake was found in both frontal



**Fig. 3. Behavioral analysis.** A. Time spent in the center in the open field test (OFT) at day 14 ( $n = 16$  per group). B. Time spent in the center in the open field test (OFT) at day 61 ( $n = 8$  per group). C. Locomotion during the OFT at day 14 ( $n = 16$  per group). D. Locomotion during the OFT at day 61 ( $n = 8$  per group). E. Floating time as a measure of immobility in the forced swim test (FST) at day 16 ( $n = 15$  per group). F. Floating time as a measure of immobility in the forced swim test (FST) at day 63 (EST + CTL  $n = 6$ ; EST + CMS  $n = 7$ ; VEH + CTL  $n = 7$ ; VEH + CMS  $n = 7$ ). G. Time that the animals attempt to escape from the cylinder, defined as highly mobile state of the animals in the FST at day 63 ( $n = 15$  per group). H. Time that the animals attempt to escape from the cylinder, defined as highly mobile state of the animals in the FST at day 63 (EST + CTL  $n = 6$ ; EST + CMS  $n = 7$ ; VEH + CTL  $n = 7$ ; VEH + CMS  $n = 7$ ). Data is presented as mean  $\pm$  SEM. Statistically significant difference are indicate by: \* $p < 0.05$ ; \*\* $p < 0.01$  and \*\*\* $p < 0.001$ .



**Fig. 4.** [ $^{18}\text{F}$ ]FDG PET Whole brain. A. Whole brain [ $^{18}\text{F}$ ]FDG uptake, as a surrogate marker of glucose metabolism, on day 13 ( $n = 16$  per group). B. Representation of changes in [ $^{18}\text{F}$ ]FDG uptake in brain regions due to short-term estrogen depletion of 13 days. C. Whole brain [ $^{18}\text{F}$ ]FDG uptake, as a surrogate marker of glucose metabolism, on day 60 ( $n = 16$  per group). D. Representation of changes in [ $^{18}\text{F}$ ]FDG uptake in brain regions due to estrogen depletion and chronic mild stress (CMS) ( $n = 8$  per group).

cortex and thalamus ( $p < 0.05$ ). In addition, the combination of OVX and CMS (EST + CMS vs VEH + CTL) induced an increase in brain metabolism in the insular cortex ( $p < 0.05$ ).

In contrast, statistical analysis of individual brain regions revealed no differences in brain metabolism due to CMS treatment alone (VEH + CTL vs. VEH + CMS or EST + CTL vs. EST + CMS). In addition, no significant interaction between chronic mild stress and estrogen depletion (EST\*CMS,  $p > 0.05$ ) was observed in any of the brain areas tested.

### 3.3.3. Within group regional metabolism changes

Within group analysis of the differences between the PET scans at day 13 and 60 showed no significant differences in the EST + CMS, VEH + CTL and VEH + CMS groups. However, a significant decrease in FDG uptake was found in the brainstem of EST + CTL when comparing day 13 and 60 (Table 4,  $p < 0.05$ ). An additional analysis, testing the effects of estradiol alone (EST + CTL and EST + CMS vs VEH + CTL and VEH + CMS) or chronic stress alone (EST + CMS and VEH + CMS vs EST + CTL and VEH + CTL) between day 13 and 60, was performed. No significant changes due to estradiol independent of CMS or vice versa were found.

## 4. Discussion

We investigated whether stress may be a trigger that aggravates depressive-like behavior in female estrogen-depleted rats, as such a

combination of stress and estrogen depletion might be responsible for the increased risk of developing depression in women going through menopause. Our results showed that a reduction in circulating estrogens increased body weight, reduced uterus and adrenal gland weight, increased depressive-like behavior and changed glucose metabolism in several brain regions. Contrary to our hypothesis, we did not find any effect of chronic stress, neither in estrogen depleted, nor in estrogen replaced animals.

In this study the OFT did not show any effect of estrogen depletion on locomotion or anxiety-like behavior. The FST, however, showed depressive-like behavior in rats after 2 months of estrogen depletion, but not yet after 2 weeks. Other studies have shown similar results, although the time interval between ovariectomy and the observed effects in the FST seems to vary among studies. Some studies found significant differences in immobility time in the FST already 1 week (Estrada-Camarena et al., 2011; Rachman et al., 1998), 2 weeks (Bekku et al., 2006; Benmansour et al., 2016; Ibrahim et al., 2016), 3 weeks (Bekku et al., 2006; Vega-Rivera et al., 2015) and 4 weeks after ovariectomy (Vega-Rivera et al., 2015). Other studies did not find any significant changes in the FST after 3 and 12 weeks (Estrada-Camarena et al., 2011) or 4-months of estrogen depletion (Fedotova et al., 2016). The differences between studies suggest that species differences, age, latency between ovariectomy and start of the estradiol treatment, type of estradiol administration, and differences in behavioral analysis could affect the response to ovariectomy-induced estrogen depletion in the FST. This complicates extrapolation of animal results to humans.

**Table 2**

Analysis of [ $^{18}\text{F}$ ]FDG PET at day 13. Changes in [ $^{18}\text{F}$ ]FDG uptake (SUV) in the brain 13 days post ovariectomy (OVX).

Decrease of [ $^{18}\text{F}$ ]FDG uptake by OVX				
Group comparison	Cluster p-value	Brain area	T-value peak level	Effect size (d-value)
EST > OVX	< 0.001	Midbrain, Thalamus, cerebellum, hippocampus, Brainstem	5.61	2.08
Increase of [ $^{18}\text{F}$ ]FDG uptake by OVX				
Group comparison	Cluster p-value	Brain area	T-value peak level	Effect size (d-value)
EST < OVX	< 0.05	Brainstem	6.24	2.32

**Table 3**

Analysis of [<sup>18</sup>F]FDG PET at day 60. Changes in [<sup>18</sup>F]FDG uptake (SUV) in the brain at 60 days post ovariectomy (OVX) and 48 days of chronic mild stress (CMS) exposure.

Decrease of [ <sup>18</sup> F]FDG by OVX effect on stressed animals				
Group Comparison	Cluster p-value	Brain area	T-value (mean ± SD)	Effect size (d-value)
EST > VEH	< 0.001	Orbitofrontal cortex, Piriform cortex - amygdala complex, Insular cortex	8.51	4.72
EST > VEH + CMS	< 0.001	Orbitofrontal cortex, Piriform cortex - amygdala complex, Frontal association cortex	7.21	4.00
EST + CMS > VEH	< 0.05	Orbitofrontal cortex, Frontal association cortex	5.71	3.17
EST + CMS > VEH + CMS	< 0.05	Orbitofrontal cortex, Frontal association cortex	6.00	3.33
	< 0.05	Orbitofrontal cortex	5.90	3.27
	< 0.05	Midbrain, Hippocampus	5.35	2.97
Increase of [ <sup>18</sup> F]FDG by OVX effect on stressed animals				
Group comparison	Cluster p-value	Brain area	T-value (peak)	Effect size (d-value)
EST < VEH	< 0.05	Hypothalamus	5.55	3.08
	< 0.05	Entorhinal cortex	5.44	3.02
	< 0.05	Entorhinal cortex	4.77	2.65
EST < VEH + CMS	< 0.001	Brainstem	6.45	3.58
	< 0.05	Entorhinal cortex	6.93	3.84
	< 0.05	Midbrain	6.90	3.83
	< 0.05	Entorhinal cortex	5.46	3.03
EST + CMS < VEH	< 0.001	Entorhinal cortex, Auditory temporal parietal cortex, Visual cortex, Hippocampus	7.70	4.27
	< 0.05	Entorhinal cortex, Auditory temporal parietal cortex	5.26	2.92
EST + CMS < VEH + CMS	< 0.01	Brainstem	7.13	3.96
	< 0.01	Brainstem	5.40	3.00
	< 0.01	Entorhinal cortex, Auditory temporal parietal cortex	4.93	2.73
	< 0.05	Entorhinal cortex, Auditory temporal parietal cortex	4.58	2.54

Besides behavioral changes, the current study showed differences in regional brain glucose metabolism due to depletion of circulating estrogen levels. Regional reductions in brain glucose metabolism after 13 days of estrogen depletion were observed in cortical and subcortical regions, and also in the cerebellum. Since no behavioral changes were observed at this stage, these metabolic changes cannot be directly linked to either anxiety-like or depressive-like behavior. Differences in [<sup>18</sup>F]FDG uptake in specific brain areas were also found after 2 months of estrogen depletion. The majority of brain regions with increased glucose metabolism as a consequence of a prolonged reduction in estrogen levels, such as insular cortex and frontal cortex, are involved in cognition and emotion, suggesting that estrogen has an effect on cognitive and executive functions (Shamungan and Epperson, 2014).

In contrast to our hypothesis, chronic stress did not aggravate the depressive-like behavior in estrogen-depleted animals in this study. The applied chronic stress protocol has been shown to induce both depressive-like behavior and anxiety-like behavior in rodents in other studies. Previous studies with CMS models have reported an increase in immobility in the FST and Tail Suspension test in female rodents (Chandrasekhar et al., 2017; Dalla et al., 2008; Franceschelli et al., 2015; Marco et al., 2017; Xing et al., 2013), and increases in anxiety levels and locomotion in the OFT, Elevated plus maze and Marble burying test (Chung et al., 2012; Franceschelli et al., 2015; Palumbo et al., 2007). There are several plausible explanations for the absence of an effect of CMS in our study. First, the animals might have become habituated to the individual stressors, since the stressful events were presented in a predictable order. Thus, animals could have experienced these events as less stressful. Second, it has been suggested that the

effect of stress is less robust in females than in males (Dalla et al., 2005; Franceschelli et al., 2014), although CMS induced differences in behavior has been observed in female rats as well (Dalla et al., 2008). Third, the high variability and contradictory results amongst the different studies could be explained by the type of stressors the duration of exposure to CMS, as CMS protocols lasting between 2 and 8 weeks have been used (Willner, 2017a, 2017b).

In contrast, Hu and colleagues found CMS-induced changes in glucose metabolism in areas like auditory and piriform cortex, and septal nuclei (Hu et al., 2010). These contradictory results may be ascribed to differences in the duration of the chronic stress, gender differences, and the types of stressors used. Studies using other chronic stress models, like chronic restraint stress (Wei et al., 2017) and chronic unpredictable stress (Lin et al., 2014; Zhou et al., 2017), have also shown changes in brain metabolism, although another study did not show any effect of chronic unpredictable stress alone on glucose metabolism (Baptista et al., 2015). Likely, differences in experimental environment, gender differences and type of stress are relevant for possible effects on brain metabolism.

Our study does not show any complementary effect of CMS on depressively-like behavior induced by estrogen depletion. A potential limitation of our study is that the CMS protocol was started 2 weeks after OVX, when estrogen levels are stably reduced. Consequently, the study design may mimic the effect of chronic stress on postmenopausal women, rather than peri-menopausal women with changing estrogen levels. By performing the CMS protocol around the day of OVX, the model will likely be better able to show the interaction between chronic stress and declining estrogen levels. In addition, more research in this

**Table 4**

Within group analysis of [<sup>18</sup>F]FDG PET. Changes in [<sup>18</sup>F]FDG uptake (SUV) in the brain between first PET scan (13 days after ovariectomy) and second PET scan (after 48 days of chronic mild stress CMS exposure).

Within group decrease of [ <sup>18</sup> F] FDG uptake					
Group	Brain area	Day 16 (average ± SEM)	Day 60 (average ± SEM)	p-value	Effect size (d value)
EST + CTL	Brainstem	2.3 ± 0.1	1.9 ± 0.1	< 0.05	1.19



field is needed to determine which other variables can be affecting the susceptibility of women to get psychiatric disorders during menopause or hormone therapy. Additional studies in animal models are also needed to increase our understanding of the central effects of the decline in circulating estrogen levels during menopause, the effect of hormonal replacement therapy, and the possible influence of chronic stress.

## 5. Conclusion

Reduced levels of estrogen as a consequence of ovariectomy can induce depressive-like behavior and changes in glucose metabolism in brain areas involved in cognition and emotion. CMS did not showed any significant contribution to the magnitude of depressive-like symptoms, or to brain metabolism in our study. However, more studies with different stress models are needed to gain more insight in the role of stress in the development of depression related to estrogen changes.

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## Declaration of Competing Interest

None.

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