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Research article

The effect of estrogen therapy on cerebral metabolism in diabetic female rats

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ABSTRACT

The impact of estrogen on brain function, especially in individuals with diabetes, remains uncertain. This study aims to compare cerebral glucose metabolism levels in intact rats, ovariectomized (OVX) rats, and 17β-estradiol (E2)-treated OVX diabetic female rats. Sixteen rats were administered a single intraperitoneal injection of 70 mg/kg streptozotocin (STZ) to induce diabetes (intact, n = 6; OVX, n = 6; OVX+E2-treated, n = 4). Additionally, 18 rats received an equivalent solvent dose via intraperitoneal injection (intact, n = 6; OVX, n = 6; OVX+E2-treated, n = 6). After 4 weeks of STZ or solvent administration, positron emission tomography scans with ^{18}F-fluorodeoxyglucose (^{18}F-FDG) injection were employed to assess cerebral glucose metabolism. The diabetic rats exhibited substantial reductions in ^{18}F-FDG uptake across all brain regions (all $P < 0.01$), in contrast to the control rats. Moreover, intact and OVX + E2-treated diabetic female rats displayed more pronounced decreases in cerebral glucose metabolism in the amygdala and hippocampus compared to OVX diabetic female rats ($P < 0.05$). These findings suggest that diabetes creates an environment wherein estrogen exacerbates neuropathology and intensifies neuronal activity.

1. Introduction

Estrogen is a pivotal hormone vital for maintaining reproductive health. Its significance, however, extends beyond reproduction, encompassing diverse functions in essential bodily processes such as cerebrovascular and cognitive functions. Notably, estrogen promotes the production of nitric oxide by endothelial cells [1], facilitating vasodilation and thereby conferring neuroprotective effects. Postmenopausal women have shown enhanced brain metabolic activity following estrogen replacement initiation, underscoring estrogen's potential in safeguarding against neuronal activity decline [2,3]. Preclinical investigations using animal models have also showcased the promising role of estrogen in conditions like cerebral ischemia [4] and neurodegenerative diseases [5]. While a wealth of evidence from clinical and preclinical studies supports estrogen's neuroprotective attributes, clinical trials have yielded divergent outcomes regarding its impact, with certain studies reporting negligible benefits of estrogen therapy on stroke [6] and cognitive function [7,8]. Given the frequent prescription of estrogen to women following surgical or natural menopause, in-depth exploration of estrogen's modulatory role in brain function remains imperative.

Evidence suggests that the neuroprotective and vasculoprotective impacts of estrogen depend on various factors, encompassing treatment protocols, physiological conditions, and comorbidities [9], where the presence of diabetes wields significant influence. Numerous animal studies have demonstrated that diabetes might hinder estrogen's vascular protective prowess [10], thus intensifying brain damage associated with strokes [11,12]. Building upon this premise, we postulated that the combined influences of estrogen and chronic hyperglycemia could potentially dampen cerebral activity, establishing an environment that heightens the brain's susceptibility to disease progression. Yet, insights into how estrogen affects cerebral activity within the context of

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the diabetic brain remain relatively sparse.

In this study, we conducted a comparative analysis of cerebral glucose metabolism levels among intact, ovariectomized (OVX), and 17β-estradiol (E2)-treated OVX diabetic female rats. We employed positron emission tomography (PET) with ^{18}F-fluorodeoxyglucose (^{18}F-FDG) injection for this purpose. Given that glucose serves as the brain's primary energy source, numerous studies have underscored cerebral glucose metabolism as a pivotal biomarker of cerebral activity [13]. The diabetic brain is characterized by a reduction in cerebral metabolism [14,15]. Our study extends the scope of diabetes-related investigations, which have hitherto primarily focused on comparing cerebral metabolism levels between control and diabetes groups. Instead, we delve into the impact of estrogen therapy on cerebral metabolism within the diabetic brain. The insights from this study hold the potential to deepen our comprehension of how estrogen operates in diverse brain conditions.

2. Materials and methods

2.1. Animal preparation

This study employed thirty-six female Sprague-Dawley rats aged 7 weeks, weighing between 180 and 215 g. These rats were provided unrestricted access to a standard rodent diet along with tap water. The research protocol received approval from the Institutional Animal Care and Use Committee of China Medical University (CMUIACUC-2022–421), and the study adhered strictly to the endorsed guidelines (https://grants.nih.gov/grants/olaw/guidebook.pdf).

Following a week of acclimatization, the animals were allocated at random to either the control group (intact, n = 6; OVX, n = 6; OVX+E2-treated, n = 6) or the streptozotocin (STZ)-induced diabetic group (intact, n = 6; OVX, n = 6; OVX+E2-treated, n = 6). Ovariectomies were performed by the vendor (BioLASCO Taiwan CO., Ltd) three days prior to shipment. The method for establishing the type 1 diabetic rodent model was derived from a previously published protocol [14,16]. STZ was purchased from Sigma Chemical Co. (St. Louis, MO, USA) and was freshly dissolved in 0.1 mol/L citrate buffer (pH = 4.5) before injection. Animals within the diabetic group received a single intraperitoneal (IP) dose of 70 mg/kg STZ, leading to pancreatic beta-cell-specific cytotoxicity. Control animals were given an equivalent volume of vehicle alone. The E2 treatment protocol was modeled on the approach proposed by Isaksson et al. [17]. E2 was initially dissolved in olive oil. OVX+E2--treated animals in both control and diabetic groups were administered an IP injection of 20 μg/kg/30 μL of E2 three times weekly for a continuous period of 4 weeks. Weekly monitoring of body weight and non-fasting blood glucose levels began subsequent to the STZ or citrate buffer administration. A glucometer (Accu-Chek, Basel, Switzerland) was used to ascertain plasma glucose concentration. Diabetic rats were identified as those exhibiting non-fasting blood glucose levels exceeding 250 mg/dL. It is pertinent to note that due to glucometer limitations, readings above 600 mg/dL were recorded as 600 mg/dL. Unfortunately, two diabetic rats in the OVX+E2-treated groups succumbed two weeks following STZ administration, resulting in four remaining rats within that group. The PET/CT measurement was conducted 5 days subsequent to the culmination of the E2 treatment.

2.2. PET experiments and data analysis

An animal NanoScan PET/computed tomography (CT) scanner (PET1225, Mediso, Budapest, Hungary) was used for the PET studies. For the PET scans, the animals were anesthetized with medical air containing 2–3% isoflurane. A dose of 0.55 ± 10% mCi of ^{18}F-FDG was intravenously injected via the tail vein to visualize brain glucose metabolism. Following a 60-minute interval for ^{18}F-FDG uptake, a 30-minute PET imaging session was conducted. Subsequent to the PET imaging, CT images were captured to account for attenuation and scattering effects, employing the following parameters: 360 projections, a voltage of 50 kVp, current of 980 μA, exposure time of 170 ms, pitch of 1, and voxel size of 250 × 250 × 250 μm^3 . All PET images acquired were utilized for subsequent analysis. Throughout the experiment, heart and respiratory rates were continually monitored, and the animals' body temperature was upheld through the use of a warm water blanket.

The PET images underwent reconstruction through a three-dimensional ordered-subset expectation maximization algorithm with four iterations and six subsets within the acquisition workspace. Assessment of cerebral ^{18}F-FDG uptake was performed employing the region of interest (ROI) methodology. PMOD image analysis software (version 4.0; PMOD Technologies Ltd., Zurich, Switzerland) was utilized to delineate ROIs for specific brain regions including the cingulate cortex, hippocampus, thalamus, hypothalamus, insula, striatum, amygdala, and accumbens. The anatomical locations of these delineated regions are presented in Fig. 1. The standard uptake value (SUV), calculated as the radioactivity concentration divided by the whole-body concentration of the injected radioactivity, was employed to quantify the metabolic activity within these brain regions.

2.3. Histological assessments

Upon completion of the PET scans, all rats underwent sacrifice and subsequent perfusion with 250 mL of fixative (4% paraformaldehyde in 0.1 M phosphate buffered saline, pH 7.4). Following fixation, cerebral tissues were preserved in 10% formalin and later embedded in paraffin wax to facilitate histological evaluation. Serial cross-sections measuring 5 μm in thickness were meticulously sliced using the microtome (LEICA RM2125 RTS), and these sections were subsequently subjected to hematoxylin and eosin (H&E) staining. To analyze the expression of glucose transporter protein 1 (GLUT1), which plays a pivotal role in influencing the uptake of ^{18}F-FDG [18], the following procedures were performed. The sections were initially subjected to antigen retrieval using citrate buffer (0.1 M, pH 6.0). Peroxidase activity was blocked using 3% H_2O_2, and non-specific binding was prevented by incubating the sections in 1% bovine serum albumin. For the bioreaction, the sections were exposed to a primary anti-GLUT1 antibody (dilution 1:200, ab115730) and kept at 4 °C overnight. Following the bioreaction, the sections were incubated with a secondary antibody, goat anti-rabbit immunoglobulin G H&L with HRP (dilution: 1:1000, ab205718), at 25 °C for 1 h. Subsequently, the sections were stained using DAB substrate and hematoxylin, and then carefully mounted using a mounting medium. The resulting images were observed and captured using an optical microscope (Axioscan 7, Zeiss, Jena, Germany).

2.4. Statistical analysis

All statistical analyses were executed using MATLAB and the results were visualized through Microsoft Excel. Data are expressed as means ± standard deviations. Paired Student's t-tests were carried out to examine weight variations within each group before and after the STZ/solvent injection. Due to the limitation of plasma glucose levels being recorded as 600 mg/dL when exceeding this value, the acquired data might not conform to a normal distribution. Thus, a non-parametric Wilcoxon test was employed to evaluate the disparities in plasma glucose levels within each group before and after the STZ/solvent injection.

For the assessment of disparities between the control and diabetes groups, unpaired Student's t-tests were conducted on corresponding animal group pairs to establish brain metabolism values. Within each estrogen regimen, a one-way analysis of variance (ANOVA) was carried out on the ^{18}F-FDG uptake values, facilitating a comparison of data from intact, OVX, and OVX+E2-treated animals. In instances where the one-way ANOVA results indicated an estrogen effect, Tukey's honest significant difference test was employed. Across all tests, statistical significance was defined as a P value of < 0.05.

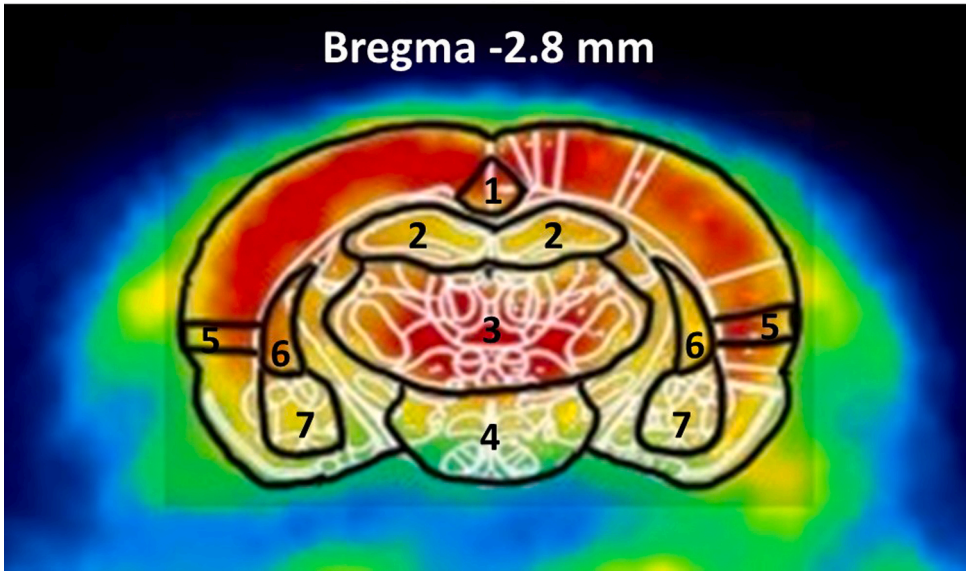

Fig. 1. Paxinos rat brain atlas warped onto a positron emission tomography image illustrating specific regions of interest. (1) cingulate cortex, (2) hippocampus, (3) thalamus, (4) hypothalamus, (5) insula, (6) striatum, and (7) amygdala.

3. Results

The weights and plasma glucose concentrations of all animals are comprehensively summarized in Table 1. Following solvent administration, the control animals demonstrated a noteworthy increase in body weight ($P < 0.05$). Comparatively, body weight was considerably higher in the OVX rats than in the intact rats ($P < 0.05$), which could potentially be attributed to weight gain as a consequence of ovariectomy [19], signifying a successful surgical outcome. Across the observation period, the plasma glucose concentration exhibited no significant changes. Conversely, in the diabetes group, animal body weights remained relatively unchanged ($P > 0.05$), while their plasma glucose concentrations displayed marked elevation ($P < 0.05$) four weeks subsequent to the STZ injection, indicative of diabetes development. It's noteworthy that the plasma glucose levels for all intact and OVX + E2-treated female animals within the diabetic group exceeded the measurable range (> 600 mg/dL), and hence were reported as 600 mg/dL.

Fig. 2 shows representative PET images of rats from both the control and diabetes groups. Visual inspection revealed consistent diabetes-related differences in brain metabolism across various brain regions, characterized by hypometabolism in the diabetic brain. Furthermore, diabetic rats with intact estrogen levels or those receiving exogenous estrogen treatment exhibited lower FDG uptake in comparison to OVX diabetic animals.

The findings from ROI analysis on estimated ^{18}F-FDG uptake across different brain regions among the groups are depicted in Fig. 3. Four weeks following STZ injection, significant reductions in ^{18}F-FDG uptake were observed in all brain regions among the diabetic animals (all $P < 0.01$) compared to the control group. Additionally, the extent of the decline in ^{18}F-FDG uptake appeared to be influenced by the estrogen regimen. Notably, intact and OVX + E2 diabetic rats displayed more pronounced decreases in cerebral glucose metabolism within the amygdala and hippocampus compared to the OVX diabetic rats ($P < 0.05$). For the control animals, the level of ^{18}F-FDG uptake exhibited no significant variation between intact, OVX, and OVX + E2 rats, signifying that the estrogen regimens had no impact on brain metabolism.

Fig. 4 shows light microscopy images of H&E-stained sections from the hippocampal dentate gyrus obtained from both the control and diabetic groups. The diabetic groups exhibited pathological changes, with neurons presenting abnormal morphologies. Most cells displayed irregular shapes, accompanied by deeply stained nuclei and nuclear condensation, indicative of neuronal injury in the diabetic group. Notably, the extent of cellular damage was more substantial in intact and OVX + E2 diabetic rats compared to OVX diabetic rats, with the H&E-stained sections also revealing a diminished overall neuronal density (highlighted by yellow arrows in Fig. 4). These H&E-stained histological findings corresponded with the results from ^{18}F-FDG uptake

Table 1
Weight and plasma glucose level measurements for animals in the control and diabetes groups.

	Control						Diabetes					
	Intact		OVX		OVX + E2		Intact		OVX		OVX + E2	
	Week 0	Week 4	Week 0	Week 4	Week 0	Week 4	Week 0	Week 4	Week 0	Week 4	Week 0	Week 4
Weight (g)	205.2 ± 0.4	247.8 ± 5.7 *	208.2 ± 5.3	276.8 ± 13.8 *	221.2 ± 7.2	277.5 ± 6.9 *	199.2 ± 4.8	189.2 ± 26.5	225.8 ± 9.7	256 ± 25.9	220.6 ± 6.4	235 ± 17.8
Glucose (mg/dL)	135.2 ± 24.7	154.6 ± 11.3	138 ± 10.4	125.7 ± 17.2	115.3 ± 5.35	129.5 ± 18.1	113.2 ± 5.1	600 @ # 0	142.2 ± 23.5	600 4 @ #	114 ± 2.58	600 @ # 0

Values are presented as means ± standard deviation.
Intact: unovariectomized female rats, OVX: ovariectomized female rats, OVX + E2: 17β-estradiol-treated ovariectomized female rats.
* : Significantly different after solvent or streptozotocin injection by using a paired Student's t-test.
@ : Significantly different after solvent or streptozotocin injection by using a non-parametric Wilcoxon test.
#: Animals with plasma glucose levels > 600 mg/dL can only be recorded as 600 mg/dL, and the data are presented as median and interquartile values.

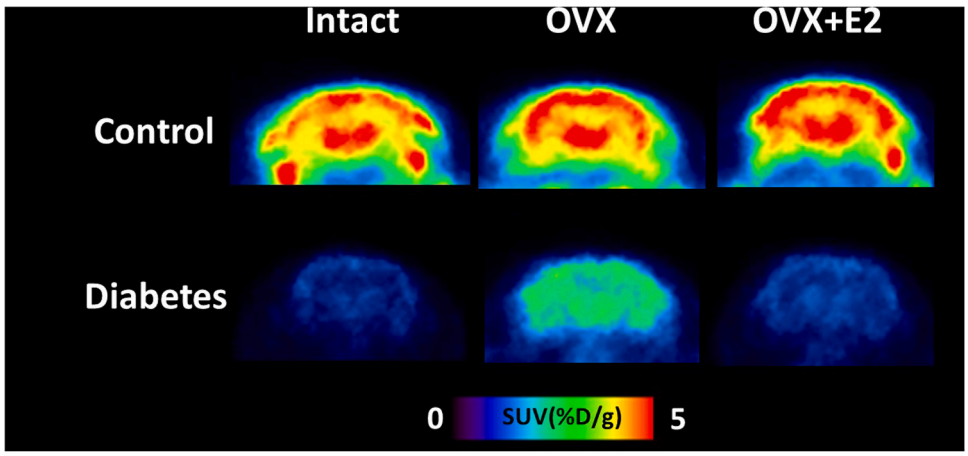

Fig. 2. Representative positron emission tomography images of cerebral ^{18}F-fluorodeoxyglucose uptake in control and diabetes groups. The anatomical locations presented in Fig. 2 are the same as that in Fig. 1. SUV, standardized uptake value; OVX, ovariectomized; E2, 17β-estradiol.

analysis.

The expression level of GLUT1 in the brain is depicted in Fig. 5. In comparison to the control group, the diabetic state significantly reduced the expression of GLUT1 level in the brain. While the levels of GLUT1 did not exhibit significant differences among the three diabetes groups, the decline in GLUT1 expression was most pronounced in the intact group and less notable in the OVX group, following the same pattern as the ^{18}F-FDG uptake in the brain.

4. Discussion

Compared to the control group, the diabetic animals displayed a notable reduction in cerebral metabolism across various regions. Furthermore, the diabetic condition seemed to alter the role of estrogen, shifting it from a neuroprotective to a neurotoxic substance. As a result, female animals with intact estrogen supply or those receiving exogenous estrogen treatment exhibited a more pronounced degree of hypometabolism. These discoveries underscore the need for clinicians to reevaluate hormone replacement therapy for diabetic patients. This is due to the realization that estrogen proves ineffective in guarding against brain changes and is linked to heightened neuronal damage and compromised brain function.

An intricate relationship is present between persistent hyperglycemia and the accumulation of advanced glycation end products (AGEs) [20,21]. AGEs themselves possess the ability to induce the generation of reactive oxygen species (ROS) by engaging with cell surface receptors known as receptors for AGEs. This interaction initiates intracellular signaling pathways that ultimately lead to ROS production [22]. This interaction establishes a self-perpetuating cycle, wherein AGEs foster increased ROS generation, consequently exacerbating oxidative stress. In the context of the vasculature, heightened oxidative stress can precipitate reduced arterial compliance and heightened vascular stiffness [23]. Consequently, the combined impact of AGEs and oxidative stress can expedite the advancement of various diabetes-related complications, such as diabetic cardiomyopathy [24] and diabetic nephropathy [25]. On the other hand, it is well-established that ROS can lead to the downregulation of GLUT1 systems [26]. GLUT1 is a protein responsible for facilitating the transport of glucose across cell membranes, playing a pivotal role in glucose uptake, especially in tissues with high glucose demand, such as the brain. Both our research and previous studies [27,28] have consistently shown that GLUT1 experiences downregulation in diabetic rats. This reduced expression of GLUT1 correlates with lower glucose utilization within the brain, a pattern consistently observed in our ^{18}F-FDG images. The pathological shifts observed in the patterns of cerebral metabolism within the diabetic brain closely resemble those documented in cases of dementia [29]. Furthermore, epidemiological data underscores an increased risk of dementia for individuals with diabetes [30]. Collectively, these observations underscore the importance of sustained follow-up to monitor cerebral changes in diabetes patients, allowing for an enhanced comprehension of the potential mechanisms underpinning the diabetes-associated decline in cognitive function.

In this study, a decrease in glucose utilization was observed in diabetic rats that were both intact and treated with OVX + E2, specifically affecting regions such as the hippocampus and amygdala. These findings contrast with previously published results that highlight estrogen's neuroprotective impact on the brain, mitigating the risk of neurodegeneration [2,3], and its role in promoting glucose utilization and enhancing metabolism [31]. In the context of diabetes, estrogen treatment might lose its advantageous effects and could potentially worsen diabetes-associated brain dysfunction, indicating the possibility of adverse consequences. Diabetes is linked to elevated levels of AGEs [20,21], and the introduction of synthetic estrogens can trigger an upregulation of the receptor for AGEs [32]. In cases of hyperglycemia, this combination of increased AGEs and estrogen-induced receptor expression could lead to heightened inflammation and oxidative stress [33], contributing to the progression of vascular complications and subsequent disruptions in glucose metabolism [34]. The distribution of estrogen receptors varies across different brain regions, with the hippocampus and amygdala exhibiting higher receptor levels [35]. Consequently, an anticipation of greater hypometabolism in the hippocampus and amygdala is warranted. These regions play a crucial role in cognitive processing [36], aligning with the present study's revelation of estrogen's detrimental effects on brain metabolism in diabetic rats. This finding resonates with clinical observations that women with diabetes who undergo postmenopausal hormone therapy are at a heightened risk of dementia and cognitive impairment [37].

The impact of estrogen on cognitive function holds a special significance, yet the body of research on this topic presents conflicting outcomes. While some studies have documented positive cognitive effects of estrogen in postmenopausal women [38,39], there are also reports of adverse impacts [7,8]. This variance in results could stem from variations in cognitive evaluation tasks employed across studies and differences in hormone therapy protocols. Furthermore, demographic diversity might also contribute to this divergence. Even though certain demographic factors like age and educational background were controlled for in previous investigations, the consideration of a diabetes history was omitted in certain cases [38,39]. Given that estrogen exacerbates neural activity and consequent cognitive impairment in individuals with diabetes, the presence of imbalanced proportions of

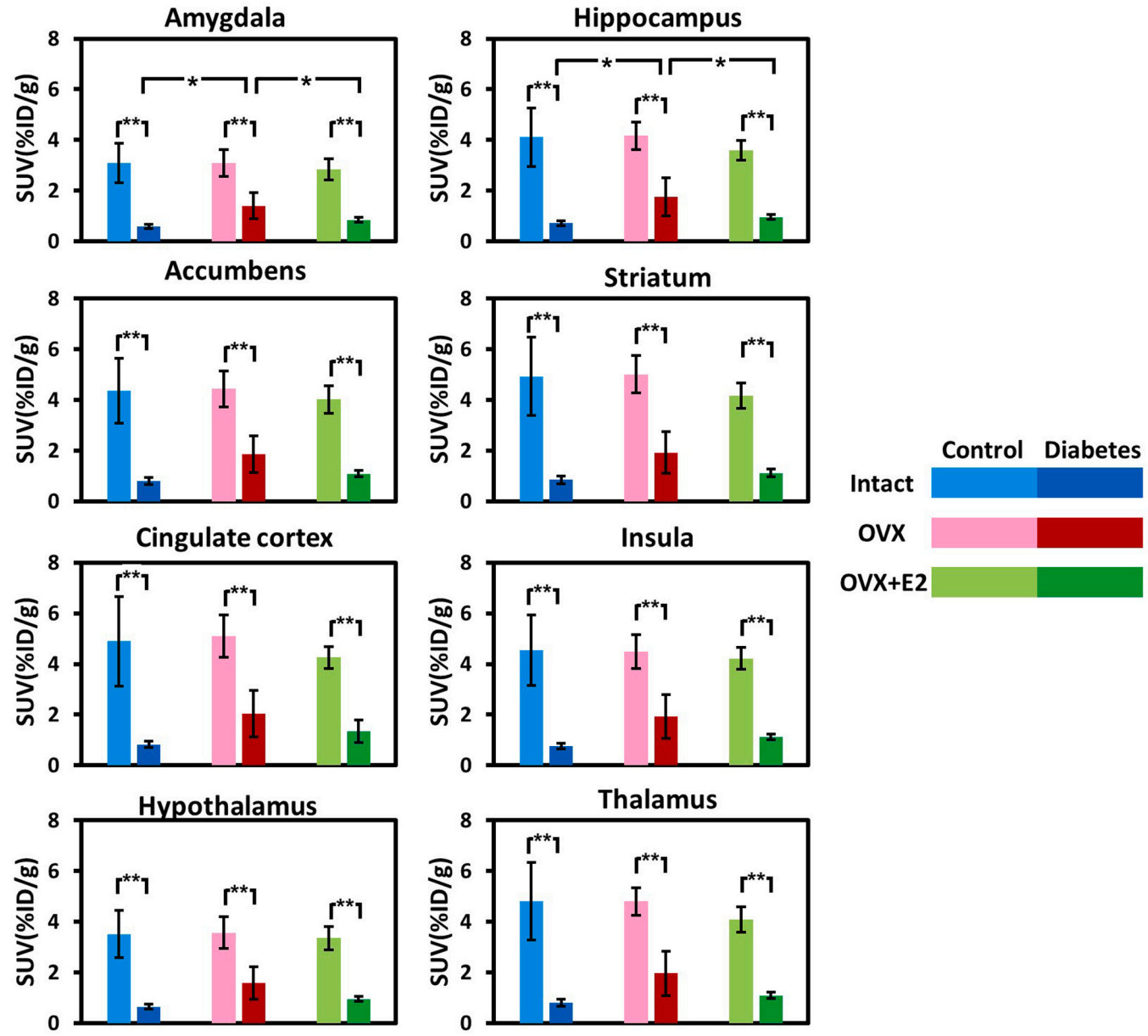

Fig. 3. Regional comparisons of ^{18}F-fluorodeoxyglucose uptake values between control and diabetic groups. * : P < 0.05; * *: P < 0.01. SUV, standardized uptake value; OVX, ovariectomized; E2, 17β-estradiol.

diabetic patients within estrogen and placebo groups could introduce biases in comparisons involving these groups. Considering that diabetes incidence rises notably with age, it becomes imperative to account for diabetes when probing the influence of estrogen on cognitive function among postmenopausal women.

Diabetes stands as a significant risk factor for stroke, and the prediction of recovery post-stroke might be facilitated by monitoring cerebral glucose metabolism. Lower cerebral glucose metabolism is closely linked to worse outcomes, emphasizing its potential predictive value [40]. Initially, this might suggest that intact and OVX+E2-treated diabetic rats would exhibit more pronounced dysfunction after stroke compared to OVX diabetic rats. Nevertheless, the outcomes of this study don't consistently align with findings from other researchers. Regarding infarct volume, OVX+E2-treated diabetic animals displayed the largest volume, while intact and OVX diabetic animals showed no significant differences [11]. Santizo et al., when assessing stroke consequences via neurological scores, reported that the severity of neurological deficits was highest in OVX + E2-treated diabetic female rats and lowest in intact diabetic female rats [12]. This diversity in findings across studies could potentially be attributed to methodological factors. Surgical preparation in animal models of cerebral ischemia, like bilateral common carotid artery occlusion, can introduce considerable stress to the animals, confounding brain metabolism influences. Anesthesia, a crucial component of these experiments, can also substantially impact outcomes. The choice of anesthesia might influence the neurological consequences of experimental stroke [41]. Given the variability in anesthesia regimens among studies, interpreting results from animal studies using different anesthetic agents demands caution. For a more comprehensive comprehension of the underlying mechanisms driving these disparities among studies, monitoring brain metabolism levels before and after stroke is recommended.

The findings of this study necessitate cautious interpretation due to inherent limitations. Firstly, the omission of plasma estrogen concentration assessment raises a concern. Adhering to Isaksson et al.'s regimen, the study aimed for plasma estrogen levels within the normal rat estrous cycle range [17], ensuring the fidelity of reported detrimental

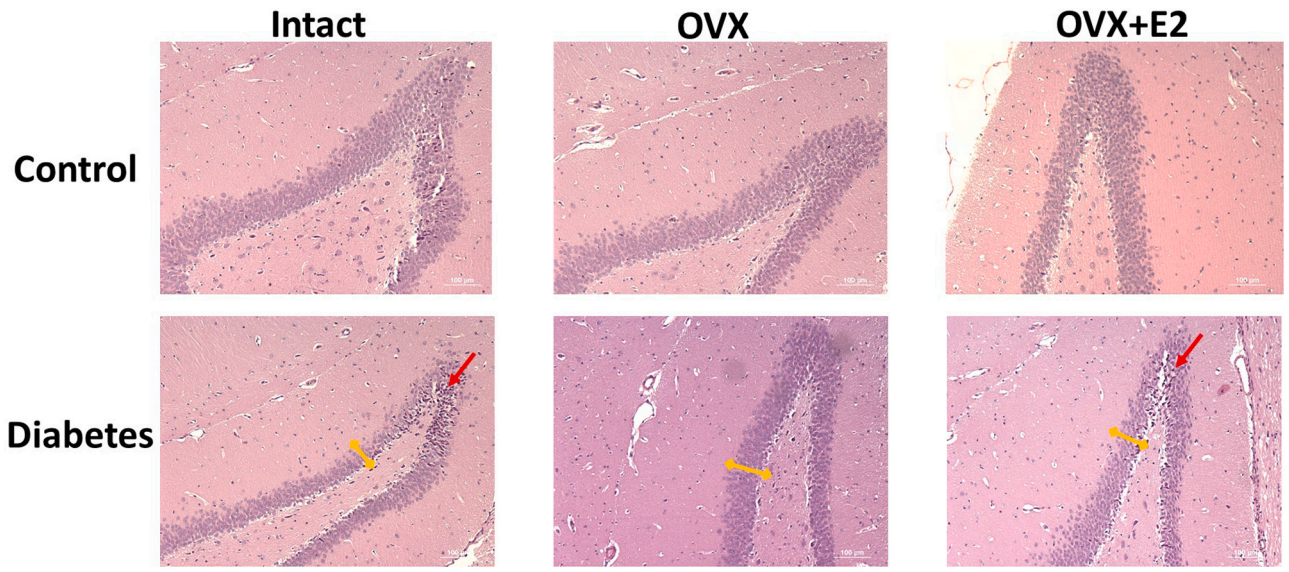

Fig. 4. Histological assessments of hippocampal dentate gyrus using hematoxylin and eosin staining. Red and yellow arrows indicate damaged neurons and decreased neuronal density in H&E-stained sections, respectively. OVX, ovariectomized; E2, 17β-estradiol.

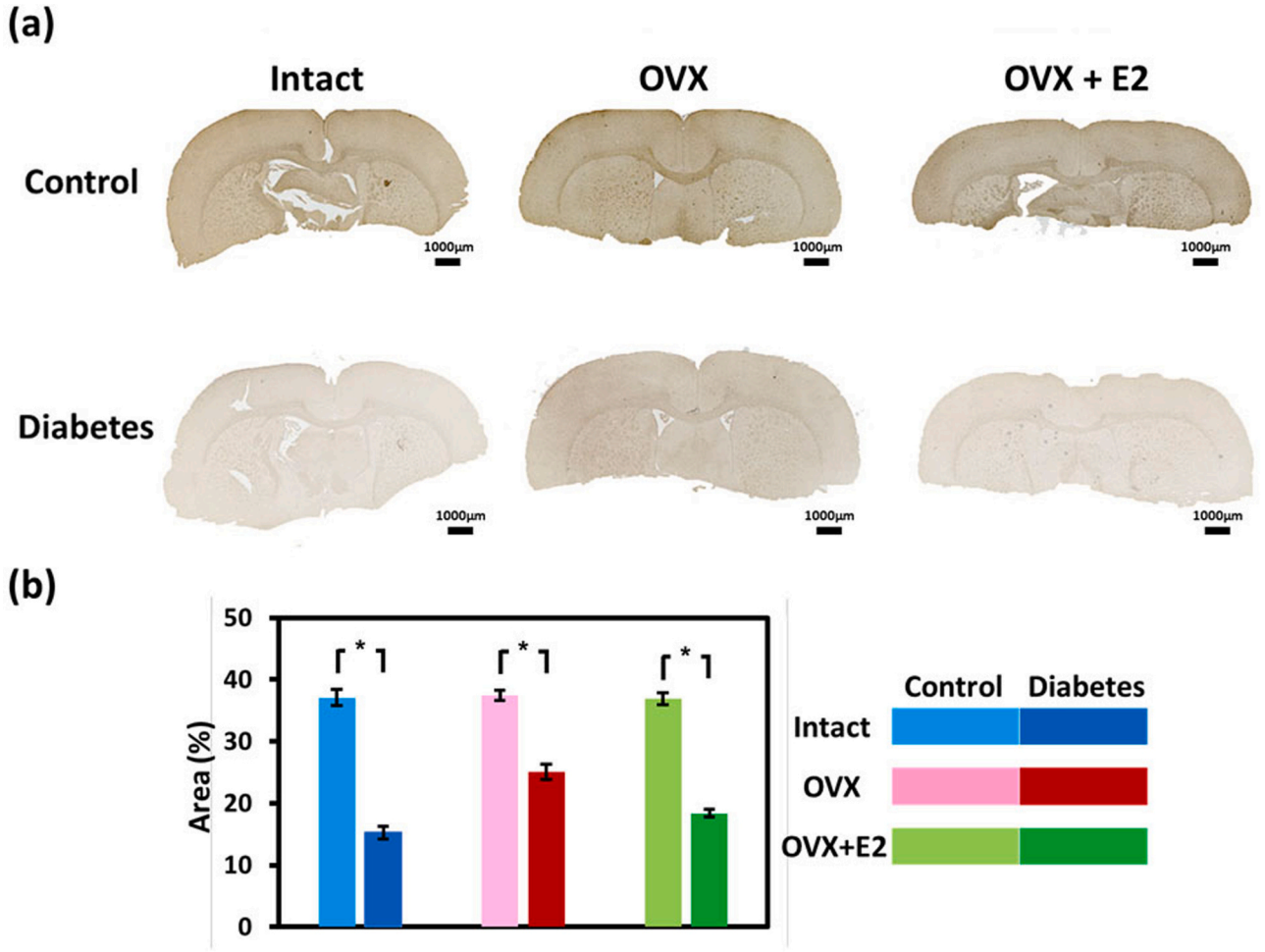

Fig. 5. Immunohistochemistry (IHC) staining of brain for glucose transporter 1 (GLUT1). (a) IHC images for the expression of GLUT1. (b) Quantitative analysis of GLUT1 expression in the six groups of rats. *: $P < 0:05$.

estrogen effects on brain metabolism in diabetes. However, future studies should prioritize monitoring plasma estrogen levels. Secondly, only animals with uncontrolled blood glucose levels and type 1 diabetes were included. While prior studies underscore a reduction in estrogen's neuroprotective impact against cerebral ischemia in hyperglycemic diabetic animals [12], it's noteworthy that estrogen treatment can mitigate ischemic injury in normoglycemic diabetic animals [42]. These insights imply a possible mediation of estrogen's effects by plasma glucose concentration. Hence, exploring diabetes-related metabolic shifts necessitates experimental designs encompassing insulin-treated and normoglycemic female rats similar to the current design. Thirdly, a restricted sample size was employed due to ethical considerations aiming to minimize animal use. Although one-way ANOVA accentuated estrogen's impact on diabetic brain metabolism, a conservative approach was adopted by reassessing data with two-way ANOVA to detect an interaction effect between diabetes and estrogen. While the two-way ANOVA yielded a non-significant but trend-indicating effect ($P = 0.15$ and $P = 0.11$ for the amygdala and hippocampus, respectively), significant interaction effects between diabetes and estrogen emerged (both $P < 0.05$), affirming distinct brain responses to estrogen in diabetic and control animals. This justifies our conclusions, yet larger participant cohorts are advisable for robust statistical analyses. Fourthly, our histological assessment exclusively utilized H&E staining and GLUT1 IHC staining to evaluate histological changes. While these staining results align with the observed ^{18}F-FDG uptake patterns, it is crucial to acknowledge that diabetes involves additional pathogenic mechanisms, including an increased presence of ROS, as elaborated upon in our discussion. To lend further credence to our hypothesis, which proposes that estrogen-induced upregulation of the receptor for AGEs might lead to the augmentation of inflammatory and oxidative stress, the utilization of more intricate and sensitive immunohistochemistry techniques becomes essential. Techniques such as 4-hydroxynonenal (4-HNE) or 8-hydroxy-2′-deoxyguanosine (8-OHdG) immunohistochemistry [43] are pivotal for precisely identifying oxidative stress within the brain.

This study demonstrates that diabetes induction causes deficits in brain glucose metabolism. Moreover, chronic hyperglycemia provides an environment in which estrogen potentiates neuropathology and exacerbates neuronal activity. The identification of idiosyncratic patterns of estrogen-related alterations in the diabetic brain may use to investigate the incidence of cognitive impairment in women after menopause.

CRediT authorship contribution statement

C.Y. W and S.L. P wrote the manuscript and obtained funding. All authors conducted the experiments and approved the manuscript.

Declaration of Competing Interest

The authors declare no conflict of interests.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.csbj.2023.09.031.

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