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Estrogen and insulin transport through the blood-brain barrier

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HIGHLIGHTS

- Acute estrogen (E2) does not alter insulin transport into the CNS in rats.
- Chronic E2 can both prevent and reverse diet-induced obesity (DIO) in rats.
- Chronic E2 does not increase insulin transport into the CNS.
- Food restriction reduces DIO and improves central insulin transport.

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ABSTRACT

Obesity is associated with insulin resistance and reduced transport of insulin through the blood-brain barrier (BBB). Reversal of high-fat diet-induced obesity (HFD-DIO) by dietary intervention improves the transport of insulin through the BBB and the sensitivity of insulin in the brain. Although both insulin and estrogen (E2), when given alone, reduce food intake and body weight via the brain, E2 actually renders the brain relatively insensitive to insulin's catabolic action. The objective of these studies was to determine if E2 influences the ability of insulin to be transported into the brain, since the receptors for both E2 and insulin are found in BBB endothelial cells. E2 (acute or chronic) was systemically administered to ovariectomized (OVX) female rats and male rats fed a chow or a high-fat diet. Food intake, body weight and other metabolic parameters were assessed along with insulin entry into the cerebrospinal fluid (CSF). Acute E2 treatment in OVX female and male rats reduced body weight and food intake, and chronic E2 treatment prevented or partially reversed high-fat diet-induced obesity. However, none of these conditions increased insulin transport into the CNS; rather, chronic E2 treatment was associated less-effective insulin transport into the CNS relative to weight-matched controls. Thus, the reduction of brain insulin sensitivity by E2 is unlikely to be mediated by increasing the amount of insulin entering the CNS.

1. Introduction

The International Diabetes Federation has estimated that 25% of adults worldwide currently have the metabolic syndrome [1]. The metabolic syndrome is frequently associated with insulin resistance, a syndrome in which the body's responsiveness to the pancreatic hormone, insulin, is reduced. Insulin's function in the CNS is of particular importance to energy homeostasis, since it suppresses hepatic glucose production [2,3], promotes glucose uptake [3] and reduces food intake via the hypothalamus and other brain regions [4–6]. Intranasal insulin, which directly enters the CNS, produces clinically-meaningful weight loss in lean men, but not in obese men with insulin resistance [6,7].

Insulin must enter the brain in order to reduce food intake and body weight. Genetic deletion of insulin receptors in the whole brain [5], or compromising the function of insulin receptors locally within the mediobasal hypothalamus [3,8], results in hyperphagia and obesity. In contrast, mice with a knockout of insulin receptors in all tissues other than the brain do not have altered body weight, despite the presence of pronounced hyperglycemia [9].

In order to enter the brain, insulin must cross the blood-brain barrier via a selective receptor-mediated transport process. Insulin transport into the CNS of rats is impaired in high-fat diet-induced obesity (HFD-DIO), but can be fully restored by dietary intervention with a low-fat diet (LFD) [10]. This observation has also been implicated in obese humans; the proportion of insulin in the brain relative to the blood is lower in individuals with greater visceral adiposity and insulin resistance [11,12]. Therefore, insulin transport through the BBB and its subsequent action in the brain are both considered important for maintaining energy balance [13].

Therapeutics that increase the sensitivity of insulin or other catabolic signals in the brain could be used to ameliorate the metabolic syndrome and avoid the associated risks of type-II diabetes, cardiovascular disease,

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and other co-morbidities [14,15]. Estradiol (E2) is a strong therapeutic candidate in this regard, since it promotes systemic insulin sensitivity [16,17] and enhances the strength of other signals that reduce food intake and body weight, including leptin [18], cholecystokinin [19,20], and apolipoprotein-A-IV [21]. In clinical and rodent models of surgical menopause, E2 has also been found to improve insulin sensitivity in humans and rodents, as assessed using a hyperinsulinemic-euglycemic clamp [22–24] and prevents visceral adiposity [25,26]. Furthermore, a recently-developed conjugate of E2 and glucagon-like peptide-1 (GLP-1) has been demonstrated to reverse the metabolic syndrome in both male and ovariectomized (OVX) female mice, while avoiding the undesirable side-effects of estrogen treatment, such as oncogenicity and reproductive endocrine toxicity [27,28]. Considering these benefits, it is surprising that E2 reduces the apparent sensitivity of insulin that has been administered into the brain of rats, despite increasing the sensitivity of leptin in the brain under the same conditions [18]. Consistent with this, human studies have also confirmed that relative to females, males are more sensitive to insulin as an anorectic signal when administered so as to act within the brain [6,29]. This raises a paradox: why does E2 improve peripheral insulin sensitivity in humans and rodents, but act oppositely in the brain [16,30]?

Although E2 acts through several signaling pathways that also play an active role in insulin signaling (e.g., PI3K/Akt and Erk), it is unlikely that E2 out-competes insulin for activation of these common pathways. Indeed, E2 cooperates and/or synergizes with leptin and several satiation signals to reduce food intake and body weight via shared signaling pathways (e.g., PI3K/Akt) in the hypothalamus and nucleus tractus solitarius (NTS) [31]. Additionally, E2 does not reduce the expression or sensitivity of insulin receptors in the brain of rodents, making it unlikely to reduce insulin signaling [18,32,33].

Insulin acts in a dose-dependent manner in the CNS; the greater the administered dose of insulin directly into the CNS, the larger the suppression of food intake and body weight, up to the point of receptor saturation [34]. E2 may therefore interact with the transport of insulin into the brain. An E2-elicited enhancement of insulin transport in females might increase brain insulin to levels that saturate brain insulin receptors, rendering females relatively less sensitive to centrally-administered exogenous insulin. Based on this possibility, we hypothesized that E2 exerts its effects on food intake and insulin sensitivity, in part, by increasing the transport of insulin into the brain. To test this hypothesis, we determined the effect of estrogen on insulin transport into the brain following acute or chronic E2-treatment of OVX and male rats after verifying the expression of estrogen receptor-α at the BBB.

Males were included in our studies, because the male brain appears to be relatively more sensitive to insulin than OVX females [18]; although OVX reduces estrogen production and increases the apparent sensitivity of the brain to insulin, they remain less sensitive to the same dose of insulin than males and an effect can only be observed at higher doses [18]. This is consistent with the observation that post-menopausal women remain insensitive to the anorectic effects of intranasal insulin, which is comparable to the effects seen in pre-menopausal women [29]. Males are very responsive to the effects of E2, which reduces the apparent sensitivity of insulin in the brain, such that they have the same response as females [18]. Therefore, if E2 is affecting insulin transport into the CNS, this effect would likely be easier to detect in males than in OVX rats. In all studies, insulin transport into the CNS was assessed in vivo using our cerebrospinal fluid (CSF) collection technique [10,35,36].

2. Materials and methods

2.1. Animals, diets, and determination of obesity

Adult female and male Long-Evans rats (14 weeks of age; Envigo, Indianapolis, IN) were housed in individual tub cages in a temperature-controlled vivarium with a 12/12-hour light/dark cycle (lights on at 0200 h). Rats were maintained on standard rat chow (Purina, St. Louis, MO) or were provided a 40% high-fat diet (HFD), as indicated, which contains butter fat as the primary lipid (Research Diets, #D03082705). Animals were provided ad libitum access to food and water, except where indicated. Following two weeks of acclimation in the facility, females were ovariectomized [37] and allowed to recover for two additional weeks prior to the study. All protocols were followed in accordance to the guidelines approved by the University of Cincinnati Institutional Animal Care and Use Committee (IACUC).

Humans with a body mass index (BMI) of greater than or equal to 30 are classified as obese by the World Health Organization. However, there is no standard definition of "obesity" in rodents [38], since the progression of obesity is highly dependent on the strain being studied [39]. Our lab has previously characterized diet-induced obesity in Long-Evans rats using the same HFD that was selected for the current study, which defined "obesity" as a 10% or greater difference in body weight relative to controls fed a LFD [40]. Therefore, we continued treatments with E2 and vehicle as long as necessary, in order to achieve this weight difference, which took approximately 5 weeks. Similar to human obesity, our weight-based definition of obesity approximates the BMI and does not account for the relative proportion of fat mass, lean mass, or metabolic parameters.

2.2. Rat brain microvessel isolation and analysis

Brain microvessels (comprising the BBB) were isolated from rat brains using an approach based on existing protocols [41,42]. After rapidly anesthetizing the rats with isoflurane, brains were harvested as previously described [37] and immediately cooled to 4 °C in M199 medium on ice for 15 min. Meninges were removed and the forebrains were then gently homogenized with a Dounce homogenizer (Bellco, Vineland, NJ). Microvessels were pelleted in a 20% Dextran solution (Sigma) via centrifugation at 4 °C (15 min at 2500 ×*g*). After careful removal of supernatant, microvessel pellets were resuspended in chilled M199 medium and filtered through 100-micron nylon mesh (Small Parts, Inc. #7050-1220-000-20). The flow-through was collected and then passed through 20-micron nylon mesh (Millipore). Microvessels were removed from the filter via brief vortexing in 1% bovine serum albumin (BSA) in M199 medium in a new conical tube. Remaining microvessels were pelleted a final time (10 min at 1000 ×*g*, 4 °C), collected, and analyzed via qPCR or Western blotting/immunohistochemistry.

The relative purity of microvessel isolates was first verified via TaqMan qPCR using a StepOne™ Plus device (Life Technologies), by screening isolates for markers of microvessel mRNA and assessing contamination from glial and neuronal mRNA (Fig. 1A) [41]. β-Actin was used as the reference housekeeping gene to determine the relative gene expression of each microvessel and brain sample. Relative purity was also assessed by visual inspection, as well as by confirming protein expression of the BBB-marker, p-glycoprotein using immunohistochemistry. A portion of the remaining microvessel samples was then directly spread onto glass slides for immunohistochemical analysis. After fixing with 4% paraformaldehyde/PBS for 10 min, the microvessels were washed 5 times, permeabilized in 0.1% Triton in PBS, rinsed 3 times in PBS, and blocked in 2% serum. Microvessels were then incubated in primary antibody for 24 h at 4 °C. After rinsing 5 times with PBS, secondary fluorescent antibodies (Cy-3 and Alexa-488, Molecular Probes, Carlsbad, CA) were added for 1 h in 1% BSA solution. This process was repeated with a different primary and secondary antibody for co-staining experiments. After rinsing 5× in PBS, slides were mounted with SlowFade® Gold reagent (Molecular Probes). 24 h after curing at 26 °C, microvessels were visualized with fluorescence microscopy (Olympus) (Fig. 1C–E). For antibody verification, microvessel lysates were analyzed via Western blotting (Fig. 1F) with previously-validated antibodies for estrogen receptor-α (ERα) (Millipore) and the insulin-receptor-β (IR-β) subunit (Santa Cruz, sc-711). As a negative control for microvessel immunohistochemistry, we processed microvessels

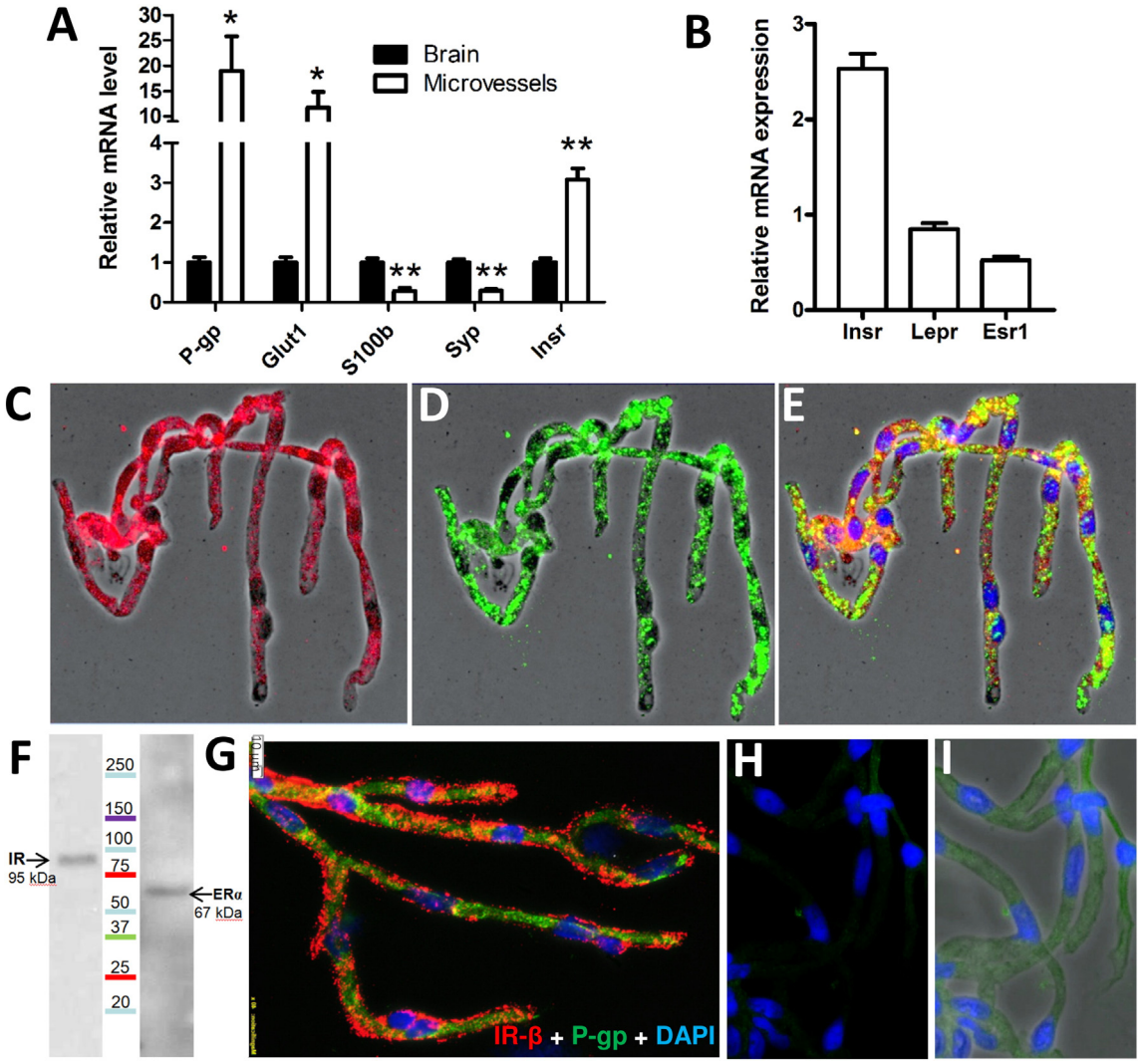

Fig. 1. The 67-kDa isoform of ERα is expressed in rat brain microvessels. The purity of microvessel isolation was confirmed via qPCR (A); P-glycoprotein (P-gp); glucose-transporter-1 (GLUT1); S100 calcium-binding protein B (S100b); synaptophysin (Syp); Insulin receptor (INSR). ERα mRNA (Esr1) was also detected in brain microvessels (B) and its expression is comparable to that of the leptin receptor (Lepr). Estrogen receptor-α (ERα) immunoreactivity was detected on freshly isolated brain microvessels (C, red/Cy3) (10× magnification). Insulin receptor-β subunit (IR-β) (D, green/Alexa-488) was expressed in brain microvessels and co-localized with ERα (E, yellow). Nuclear staining with DAPI is observed in blue. The specificity of immunoreactivity against ERα and IR-β (IR) was verified via Western blotting (F). IR-β was also co-stained along with the BBB-specific marker, P-glycoprotein (P-gp) (G) (40× magnification). As a negative control, microvessels were co-stained for Cy3 and Alexa-488 fluorescent antibodies after being incubated without primary antibodies (H–I). Fluorescent staining is negligible in these samples. n = 4, *P < 0.05, and **P < 0.005. +/− SEM.

side-by-side with other samples, except that no primary antibodies were added to the incubation solution. Microvessels were then incubated with secondary antibodies and were visualized, as described (Fig. 1H–I).

2.3. Cerebrospinal fluid collection

Cerebrospinal fluid (CSF) was collected from the cisterna magna of rats as previously described [10,35]. Briefly, rats were fasted overnight prior to the study, as indicated. Rats were then administered intraperitoneal (ip) injections of insulin during the middle of the light cycle [10,36] to maintain consistent levels of plasma insulin among groups, and CSF was collected 30 min later. After the ip injection of insulin, each rat was placed back in its home cage for 15 min prior to being anesthetized with ketamine/xylazine (55.6 mg/kg and 8.8 mg/kg, respectively). Rats were then placed into a stereotaxic instrument with the head maximally ventroflexed. A 25-G needle, with a tip prepared at a 30° angle [35], was inserted into an electrode holder and connected to microrenethane tubing filled with sterile saline. The rat's scalp and neck were shaved, the site was sterilized, and an incision was made to expose the atlanto-occipital membrane. A needle was then carefully inserted into the cisternum magnum to collect CSF beginning exactly 30 min following insulin injection. The 30-min time-point of CSF collection has been demonstrated in previous studies to be ideal for measuring the appearance of insulin in the CSF in both chow-fed and HFD-fed rats [10,36]. Using a 1-mL syringe, slight negative pressure was applied to the tubing attached to the needle for 1–2 s to initiate the flow of CSF into a chilled 1.5-mL microcentrifuge tube. Approximately 200 μL of CSF was collected in 2–3 min and was immediately frozen on dry ice. Following CSF collection and decapitation, blood was immediately collected in chilled EDTA-coated tubes and plasma was isolated. Potential blood contamination of CSF samples was measured via spectrophotometry, as described [43], by comparison to frozen, diluted rat-blood standards. Samples with > 0.001% blood contamination were excluded from analysis.

CSF and plasma samples were analyzed with an ultrasensitive rat insulin ELISA kit (Crystal Chem, Downers Grove, IL, #90060). Since the low-range assay reliably detects insulin concentrations between 0.1 and 6.4 ng/mL, we loaded 20 μL of each CSF sample per well, according to the kit instructions [36]. The resulting absorbance readings fell within the middle range of the standard curve. Values were then divided by 4 to adjust for the extra volume used, relative to the 5-μL volumes of the insulin standards.

2.4. Acute E2 treatment in ovariectomized rats

A once-daily injection of estradiol-3-benzoate (E2; 10 μg/kg, Sigma) or vehicle (20 μL, sesame oil, Sigma) was administered subcutaneously (sc) to OVX rats for 2 days, a treatment regimen previously found to exert catabolic effects in OVX rats [22]. During the 2-day treatment period, food was removed to eliminate the variable of differential food intake between groups. Using this protocol, insulin was injected 30 min prior to CSF collection after 2 days of E2 or vehicle administration.

In a parallel study, brain microvessels were isolated from a separate cohort of OVX rats and examined via qPCR for the expression of the insulin receptor, as described above. Preparation of these OVX rats mirrored the original cohort, except that insulin was not administered prior to brain-harvesting.

2.5. Acute E2 treatment in male rats

Because the effect of E2 on insulin transport may be blunted in OVX rats relative to males (as explained above) [18,44], we proceeded to use males in all subsequent studies. A single sc injection of E2 (10 μg/kg) or vehicle (20 μL) was administered to 14-week-old male rats 24 h prior to ip insulin and CSF collection. Both groups were pair-fed, by providing a fixed, limited quantity of chow at the onset of dark, in order to normalize food intake across all rats. The amount of food was pre-determined based on pilot studies involving the injection of E2 or vehicle in ad-libitum-fed rats. Food was completely consumed at least 4 h prior to CSF collection.

2.6. Chronic treatment with E2

Male rats were treated with cyclic sc injections of E2 (10 μg/kg) or vehicle (20 μL) every 4th day for a period of 5 weeks, in order to achieve plasma levels of E2 that target the physiological range of the female estrous cycle [21,22,37]. Both control rats and pair-fed rats received vehicle injections.

In the first cohort, male rats were maintained on HFD for 10 weeks to induce HFD-DIO prior to treatment with E2 or vehicle. Due to the anticipated reduction of body weight in rats receiving E2, a pair-fed group given vehicle was included to match the body weight of E2-treated rats. Thus, HFD-DIO rats were randomly divided into three groups: E2, vehicle, and pair-fed (PF). All groups were then treated with cyclical E2 while remaining on the HFD for 5 additional weeks prior to CSF collection, to promote at least a 10% lower body weight than controls [40]. Two d prior to CSF collection, rats were treated with a final sc injection of E2 or vehicle. Insulin was injected ip at a dose of 1 U/kg, 30 min prior to CSF collection.

A separate cohort of male rats was prepared in a manner that paralleled the previous study, except that treatment with E2 or vehicle was started at the onset of HFD-feeding in order to prevent HFD-DIO. Again, both control rats and pair-fed rats received vehicle injections.

After 5 weeks of treatments and HFD-feeding, CSF was collected. Again, a relative prevention of obesity in these animals was pre-defined as having at least a 10% lower body weight than controls [40]. Fasting glucose and body composition analysis were assessed 10 d prior to ip insulin and CSF collection, with E2 being injected 2 days prior to the test. Following CSF collection, whole fat pads were removed from white adipose tissue depots. Epididymal, mesenteric, retroperitoneal, and inguinal fat pads were weighed prior to freezing.

2.7. Analysis of insulin transport

Insulin transport was assessed in terms of the absolute CSF insulin concentration, as well as the ratio of CSF/plasma insulin. This ratio provides an adjusted measure of insulin in the CSF and accounts for the possibility that higher/lower insulin concentrations in the bloodstream after ip injection could lead to a higher/lower concentration of insulin in the CSF at a dose of insulin that does not saturate insulin receptors at the BBB [10].

Similarly, we performed a correlational analysis of insulin transport among groups by conducting a linear regression of plasma vs. CSF insulin. This approach determines whether higher/lower plasma insulin concentrations are associated with higher/lower CSF concentrations, indicative of effective transport. Previous studies that utilized Pearson analysis to assess insulin transport into the CNS following a 1 U/kg-dose of insulin (which targets postprandial levels of insulin in the blood) found that rats maintained on a low-fat diet had a significant correlation between CSF and plasma insulin levels, whereas no significant correlation was observed in rats maintained on a HFD [10]. In our

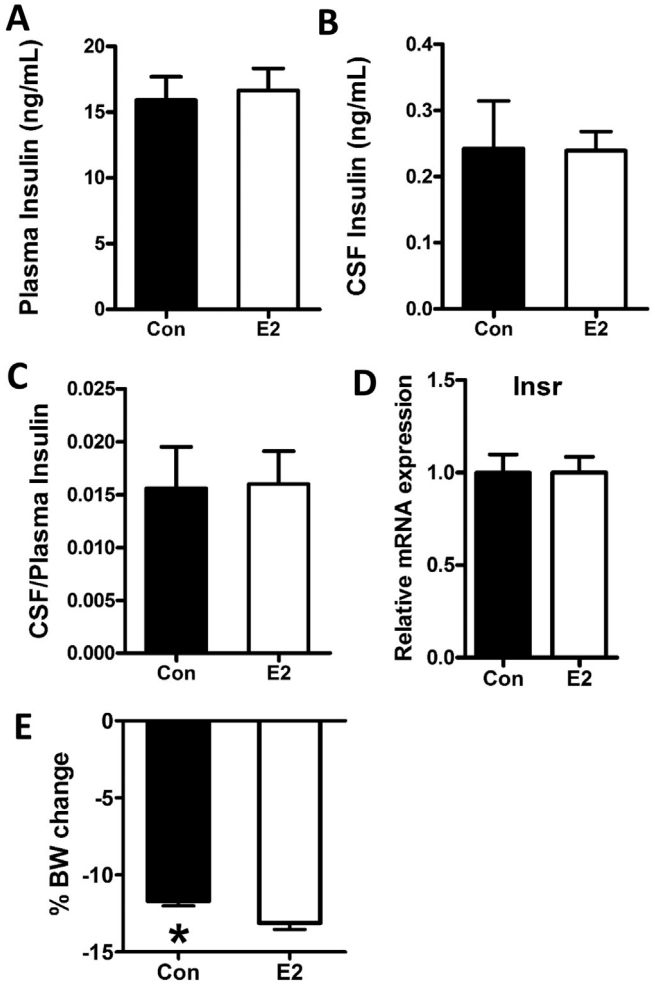

Fig. 2. Acute treatment of E2 in chow-fed OVX rats does not affect insulin transport through the BBB. Similar plasma levels of insulin were detected between control (Con) and E2-treated (E2) OVX rats, 30-min after a 2 U/kg injection (A). There was no difference in CSF insulin (B) or in the ratio between insulin levels in the CSF and plasma (C). Brain microvessel mRNA from a different group of identically-treated OVX rats was analyzed via qPCR for the relative expression of the insulin receptor (Insr) (D). The acute E2-treatment regimen led to a significant reduction in body weight relative to controls (E). n = 7–8, *P < 0.05, and +/− SEM.

current studies (Figs. 4 and 5), the plasma concentration of insulin was increased to a level that was below the saturation point of insulin transport through the BBB, as assessed in time-course studies [10,36]. Variations of the plasma insulin concentration in each rat therefore will correspond with slightly different rates of insulin transport into the CNS of each rat [36]. For example, if E2 does not affect insulin transport through the BBB, the CSF concentration of insulin will be relatively constant, regardless of the concentration of insulin in the blood. However, if E2 improves insulin transport on a HFD, then rats with higher plasma insulin would be expected to have higher CSF insulin levels, whereas controls would not. This relationship was assessed by examining the strength and significance of the Pearson coefficients among groups.

In the first two CSF-transport studies examining acute E2 treatment (Figs. 2 and 3), a high dose of insulin (2 U/kg) was used in order to saturate transporters at the BBB and to thereby maximize insulin transport [36]. In this case, variations of blood insulin do not vary in proportion to CSF transport, preventing the use of regression analysis [36]. Therefore, Pearson analyses were only conducted in rats injected with 1 U/kg insulin.

2.8. Glucose measurements

After an overnight fast, rats were anesthetized briefly with isoflurane (Isothesia™, Butler Schein) and tail tips were clipped for blood measurement. After 2 h, 300–400 μL blood was withdrawn from the tail of each rat, following removal of any scabs, and fasting blood glucose was measured in duplicate using a glucometer (Accu-Chek, Roche, Indianapolis, IN). For the insulin tolerance tests, glucose was measured from plasma samples collected 30 min following ip insulin injection, using an Analox GM7 analyzer (Analox Instruments Limited, London).

2.9. Body composition analysis

For chronic E2-treatment studies, the rats' body compositions were assessed with a rodent magnetic resonance imaging (MRI) machine (EchoMRI, EchoMedical Systems, Houston, TX). Fat mass, lean mass, and water mass were measured and body weight was also assessed at this time. In order to determine the body fat percentage, we divided each rat's fat mass by its body weight. The same calculation was used for the lean mass and water mass. No differences in water mass or percent water mass were noted, so are not reported here.

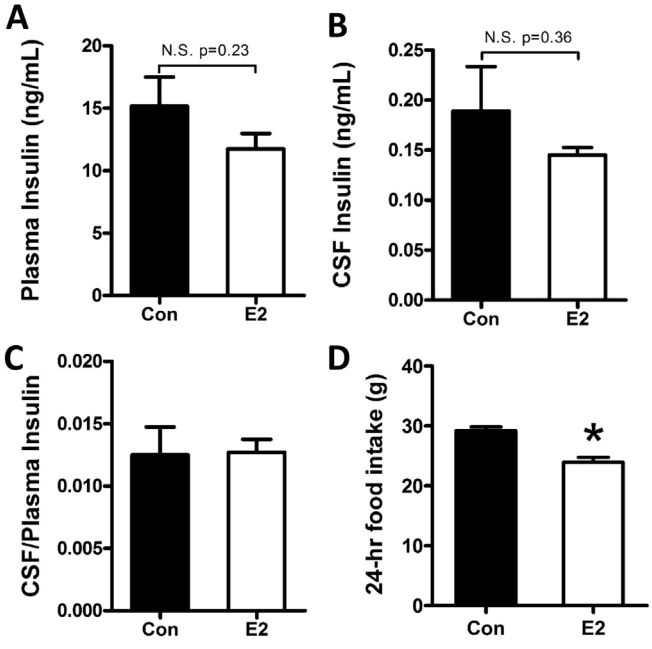

Fig. 3. Acute treatment of E2 in chow-fed male rats does not affect the transport of insulin through the BBB. No differences were observed in plasma insulin (A), CSF insulin (B), or the ratio of CSF to plasma insulin 30 min following ip insulin. A single sc injection of E2 in ad libitum chow-fed male rats acutely reduced food intake relative to vehicle-injected controls (D). n = 8, *P < 0.05, and +/− SEM.

2.10. Analysis of insulin degrading enzyme (IDE) expression in the hypothalamus and cortex

Following CSF collection in rats that underwent chronic E2 treatment, brains were collected and immediately flash-frozen. After dissection of the hypothalamus and the cortex, protein lysates were prepared and protein was quantified. After running SDS-PAGE and transferring to an Immobilon PVDF membrane (Millipore), Western Blots were incubated with primary antibody for insulin degrading enzyme for 14 h at 4 °C (1:1000, Abcam, San Francisco, CA). Blots were rinsed and incubated in horseradish peroxidase-conjugated goat anti-rabbit IgG antibody which was applied for 1 h at room temperature (1:10,000, Dako #P0448). After rinsing, Immobilon® HRP substrate (Millipore Cat. # WBKLS) was applied for 5 min at room temperature and blots were visualized on photolithography film (Denville Scientific, #E3018), scanned, and quantified using ImageJ software (NIH). After gently stripping blots for 15 min at 37 °C, membranes were rinsed and re-blotted with a loading control, the actin 5γ subunit (mouse IgG, Millipore) using similar procedures.

2.11. Statistical analysis

All statistics were analyzed using GraphPad Prism 5 using 2-way ANOVA with a Tukey post-hoc test for multiple comparisons, where applicable. Pearson analysis was conducted along with multiple linear regression, as described in Section 2.7, to determine the strength and significance of the correlations between insulin concentrations in the plasma and CSF. In all studies, we pre-defined our cutoff for statistical significance at $P \leq 0.05$.

3. Results

3.1. Verification of estrogen receptor-α expression at the BBB

As depicted in Fig. 1A, the brain microvessel isolates were highly enriched for P-gp (p-glycoprotein) and Glut-1 (glucose transporter-1) mRNA, which are highly expressed in brain endothelial cells relative to whole brain homogenates [41]. Expression of the glial cell marker, S100b (S100 calcium-binding protein B), and the neuronal cell marker, Syp (synaptophysin) mRNA was significantly lower in the BBB-isolates relative to the whole-brain homogenates, indicating that the isolation process enriched brain microvessels while selecting against neurons and glia [41]. Insulin receptor mRNA expression was elevated 3-fold in microvessels relative to the whole-brain homogenate. This finding was also reflected at the protein level (data not shown). Since insulin cannot passively diffuse through the BBB [45] and is understood to rely on an insulin receptor-mediated transport process in brain endothelial cells [46], the enrichment of insulin receptor expression in microvessels provides further evidence that the insulin receptor has a direct role in insulin transport through the BBB. We also verified microvessel purity at the protein level via immunohistochemistry for p-glycoprotein (P-gp), which is considered a marker of the BBB [41], due to its high enrichment in brain microvessels relative to the rest of the brain [47,48] (Fig. 1G). Microvessels that were processed without the addition of primary antibodies were devoid of nonspecific binding from the secondary fluorescent antibodies (Fig. 1H–I).

Since estrogen receptor-α (ERα) is the most pertinent E2 receptor involved in the regulation of food intake and body weight, we first determined that ERα is expressed at the BBB. ERα was detected at the mRNA level in freshly-isolated microvessels, with comparable

expression as the leptin receptor (Fig. 1B). Insulin receptor mRNA expression was approximately 3-fold higher than that of ERα. ERα immunoreactivity was also detected in freshly-isolated brain microvessels from both male and OVX female rats and appeared to be ubiquitously expressed in the microvessels (representative staining in Fig. 1C). We also detected abundant protein expression of insulin receptor (Fig. 1D), as expected [45,46], which co-localized with ERα (Fig. 1E). The specificity of the ERα and IR-β antibodies was verified with Western blotting of brain microvessel lysates (Fig. 1F). Single bands corresponding to the expected sizes of IR-β and ERα were observed. These findings collectively established an anatomical basis for an interaction of E2 with insulin transport at the BBB.

3.2. Effects of short-term E2-treatment on BBB-insulin transport in OVX rats

While E2-treatment of OVX rats significantly enhanced weight loss relative to control rats under these conditions (Fig. 2E), no difference in the appearance of insulin in the CSF was observed 30 min after ip insulin (Fig. 2B). The plasma-to-CSF insulin ratio also did not differ among groups (Fig. 2C). Microvessels from OVX rats treated with acute E2 or vehicle injections also had comparable expression of insulin receptor (Insr) mRNA (Fig. 2D).

3.3. Effects of short-term treatment with E2 on BBB-insulin transport in male rats

Consistent with the effects observed in OVX rats, acute E2 treatment did not alter the appearance of insulin in the CSF of males (Fig. 3B–C). The efficacy of acute E2 treatment in males was verified in pilot studies, which demonstrated that food intake was significantly reduced relative to vehicle-treated controls (Fig. 3D). Thus, acute E2 treatment, while having its expected effect on food intake and body weight, did so without altering the expression of insulin receptors in the BBB and without eliciting a change of insulin transport into the CNS.

3.4. E2 reverses HFD-DIO without improving insulin transport through the BBB

We next addressed the possibility that E2 requires a longer treatment period to exert detectable effects on insulin transport at the BBB. Previously, we found that male rats with HFD-DIO have impaired transport of insulin into the CSF relative to controls on a LFD, and that this impairment was reversed by dietary intervention with LFD [10]. Since systemic E2 both reverses HFD-DIO [27] and increases systemic insulin sensitivity in rodent models [17,49], we determined whether these

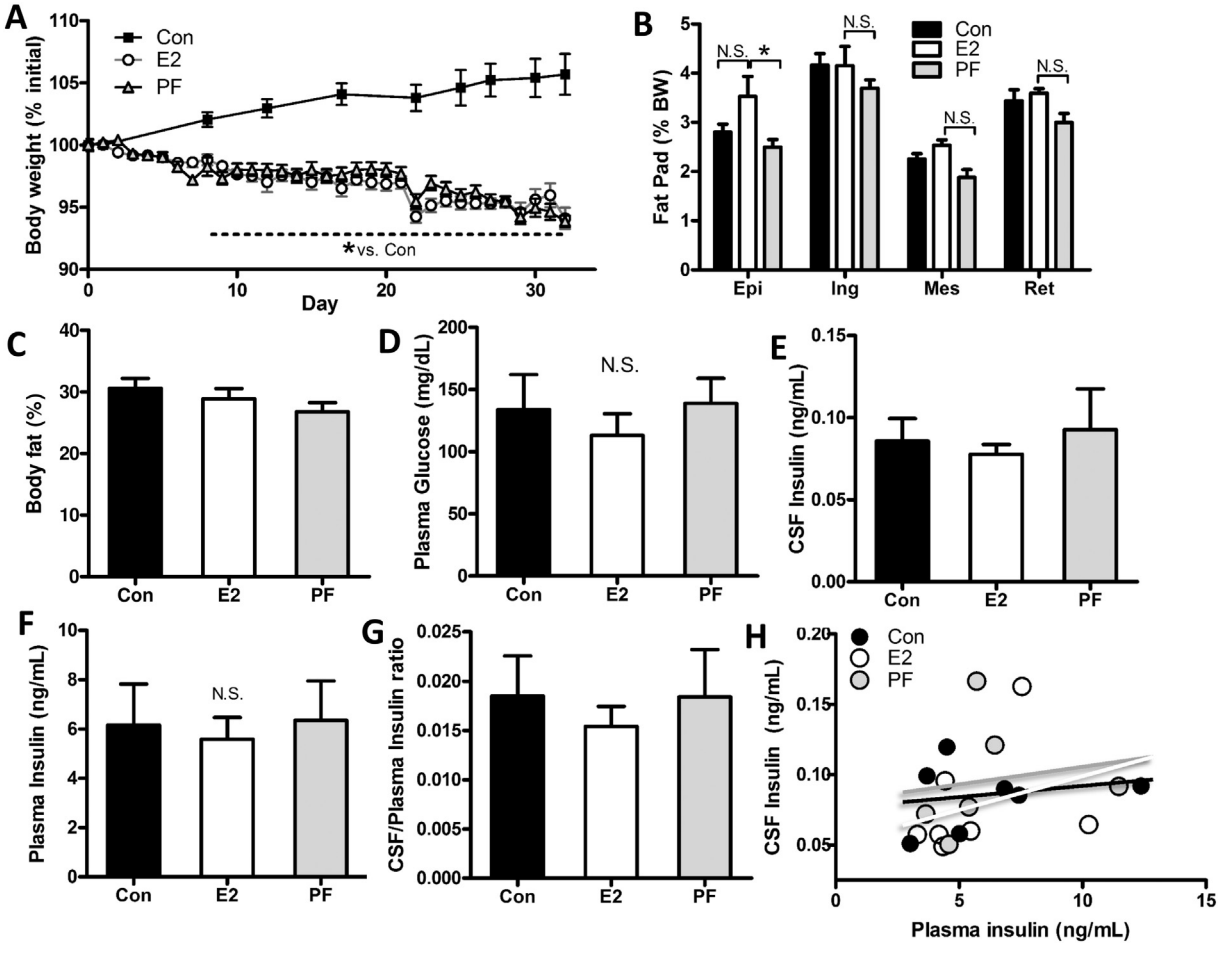

Fig. 4. E2 reverses HFD-DIO in male rats independently of the BBB-insulin-transport system. (A) 24-week-old HFD-DIO male rats were treated with E2 or vehicle (Con) with ad-lib access to HFD or were pair-fed (PF) to the body weight of E2-treated rats for 33 days. (B) Fat pad masses of the rats were normalized to the total body weight to calculate the percent weight of each pad; Epi: epididymal, Ing: inguinal, Mes: mesenteric, and Ret: retroperitoneal. Body fat percent (C) was relatively constant during the treatment period. Plasma glucose was measured 30 min following ip insulin (D). Plasma insulin levels were comparable among all groups after 30 min following ip insulin (E). CSF insulin levels were assessed (F) and the ratio of CSF to plasma insulin did not differ among groups (G). Pearson analysis of the concentrations of plasma insulin vs. CSF insulin did not reveal any significant correlations (H) and lines were not significantly different from one another ($P = 0.884$), indicating that plasma insulin levels were not associated with increased insulin transport in all animals. The y-intercepts of the lines were also not significantly different from one another ($P = 0.705$). Even when all data points were combined, the slope of the resulting line was still not significantly different from zero ($P = 0.312$). $n = 6–7$, $^*P < 0.05$, and +/− SEM.

metabolic improvements are partly mediated by chronically improving insulin transport through the BBB.

As expected, cyclic E2 treatment led to a significant reduction in body weight (12%) relative to vehicle-treated controls by the end of the 5-week treatment period (Fig. 4A). Pair-fed rats were effectively matched to the body weight of E2-treated rats. Because visceral adiposity is associated with impaired insulin transport through the BBB [10], we also examined the influence of visceral adiposity per se. E2 and pair-feeding reduced total body fat relative to controls (data not shown), but did not change the percentage of body fat (Fig. 4C) or of lean mass (data not shown), indicating that the loss of body weight resulted from a proportional loss of fat and lean tissue. There was also no relative change in the percent fat pad mass of inguinal fat (subcutaneous) or retroperitoneal and mesenteric fat (visceral), among all groups (Fig. 4B). Interestingly, although PF rats had a comparable proportion of epididymal fat as controls, E2-treatment significantly increased the proportion of epididymal fat relative to PF rats (Fig. 4B). In addition, all rats had comparable fasting blood glucose levels (data not shown) and there was no difference in the levels of plasma glucose 30 min after ip insulin injection despite the significant difference in body weight and epididymal fat (Fig. 4D).

CSF insulin levels were not significantly altered by E2 treatment relative to controls (Fig. 4E), despite comparable levels of insulin in the blood among groups in this paradigm (Fig. 4F). An additional analysis, comparing the ratio of insulin in the CSF relative to the level of insulin in the blood, yielded no difference among treatments (Fig. 4G).

Because saline-injected groups were not included to control for potential differences in the baseline insulin levels in the CSF, we conducted an additional correlational analysis to identify differences in insulin transport among groups. Since there was variability in the levels of plasma and CSF insulin 30 min after ip insulin, we correlated the concentration of plasma insulin with the concentration of CSF-insulin that was detected at the same time-point. No significant associations were observed with Pearson analysis; both the slopes of the lines and y-intercepts were not different among groups, such that lines were indistinguishable from each other. Since groups were no different from each other, all points were combined into a single correlational analysis. The Pearson correlation of all groups combined resulted in a slope that was still not significantly different from zero, indicating that the concentration of insulin in the CSF was independent of the concentration of insulin in the blood.

3.5. E2 prevents HFD-DIO and protects systemic insulin sensitivity, but does not affect insulin transport through the BBB

Although E2 was unable to reverse a pre-existing impairment of insulin transport through the BBB, it is still possible that E2 may influence BBB-insulin transport by HFD-DIO in younger rats (14-week-old males treated with E2 or vehicle immediately at the onset of HFD-feeding for 5 weeks). E2 readily prevents HFD-DIO and improves insulin sensitivity in a similar paradigm [17,33].

E2-treatment significantly prevented weight gain over the 5-week HFD-feeding period (Fig. 5A). Fat mass and lean mass were significantly reduced, but E2 did not alter the total percentage of body fat (Fig. 5C) or lean mass (not shown) relative to controls. However, PF rats did have a significant reduction in the proportion of body fat (Fig. 5C), while the proportion of lean mass was increased in comparison to controls (not shown). The proportion of visceral and subcutaneous fat pads did not differ between E2-treated and control rats (Fig. 5B). However, PF rats had a significantly smaller percentage of fat in visceral fat pads compared to E2-treated and control rats and also had a smaller percentage of fat in the inguinal fat depot (a subcutaneous fat pad) relative to E2-treated rats (Fig. 5B). Together, this indicates that E2-treatment reduced body weight by evenly reducing the proportion of fat and lean mass, whereas the reduction of body weight by pair-feeding resulted in a modest shifting of fat mass to lean mass.

While fasting glucose was not significantly different between E2-treated and PF rats (Fig. 5D), plasma glucose levels were significantly reduced in E2-treated and PF rats 30 min following ip insulin (Fig. 5E). In contrast, control rats did not have significant changes in plasma glucose after insulin injection, confirming that these rats were insulin-resistant. The comparable effects observed between E2-treated and PF rats indicate that in this paradigm, E2 mediates its effects on glucose homeostasis and insulin sensitivity via a reduction in body weight, but not via a reduction in body adiposity.

30 min after ip insulin, no difference in the concentration of CSF insulin was observed among groups (Fig. 5G). Plasma insulin levels were also not statistically different among groups (Fig. 5F), and the ratio of insulin between the CSF and plasma compartments also did not differ among groups, which accounts for small variations in plasma insulin among groups (Fig. 5H).

Linear regression analysis revealed a significant Pearson correlation for the PF group, but not for the E2-treated or control rats (Fig. 5I). The best-fit line of the PF group was also significantly different from the lines of the E2-treated and control rats, suggesting that pair-feeding is associated with a modest protection of BBB-insulin transport against the impairment of HFD-DIO, despite no detectable differences in absolute CSF insulin levels among groups. In contrast, E2-treated rats did not differ from controls based on this analysis.

To further verify that BBB-insulin transport is not changed by E2 treatment, we measured insulin-degrading enzyme (IDE) in the brain. Previous reports have highlighted the possibility that E2 may upregulate the expression of the IDE in the brain under certain conditions [50], which could increase the degradation of insulin and reduce insulin levels in the CSF. This could potentially counteract an elevated rate of BBB-insulin transport if E2 increases transport but also increases IDE expression. We therefore measured the expression of IDE in the hypothalamus (Fig. 5I) and cortex (Fig. 5J) via Western blotting, and found that the expression of IDE was not altered by E2 treatment or pair-feeding, relative to controls, further strengthening the conclusion that E2 does not exert its catabolic or systemic metabolic actions by altering insulin transport into the CNS.

4. Discussion

Our finding that ERα is abundantly expressed in rat brain microvessels is consistent with previous reports examining ERα in rat [51] and human [52] cortical endothelial cells. Our studies are unique in revealing strong expression of the primary, 67-kDa ERα isoform, which is enriched in the ovaries, muscle and brain [51], and which has a strong link with energy homeostasis [53]; and importantly, ERα was co-expressed in the same BBB cells as the insulin receptor. Overall, these findings provided a basis for a potential intracellular interaction between E2-ERα signaling and insulin transport at the BBB.

Acute E2 treatment did not affect insulin transport in OVX or male rats, despite significantly reducing body weight and food intake, respectively, relative to controls (Fig. 2A–C and Fig. 3A–C). Similarly, under conditions of chronic E2 treatment, insulin transport was not increased after the prevention or reversal of HFD-DIO (Fig. 4E–G and Fig. 5F–H), even though insulin sensitivity was significantly protected in males treated with E2 at the onset of HFD-feeding. Because systemic insulin sensitivity is positively associated with insulin transport through the BBB [10,54], it is surprising that E2-treatment did not affect insulin transport into the CNS under these conditions. However, since systemic insulin resistance was not reversed in HFD-DIO male rats, despite significant loss of body weight over the 5-week period (Fig. 4A, F), it is less surprising that insulin transport was not improved in these animals.

It is possible that the failure to detect a difference of insulin transport following chronic E2-treatment and HFD-feeding was due to differences in the levels of insulin in the blood and CSF. Because control rats maintained on HFD are insulin resistant relative to E2 or PF groups (Fig. 5E), it is possible that controls started with slightly higher levels of CSF-insulin

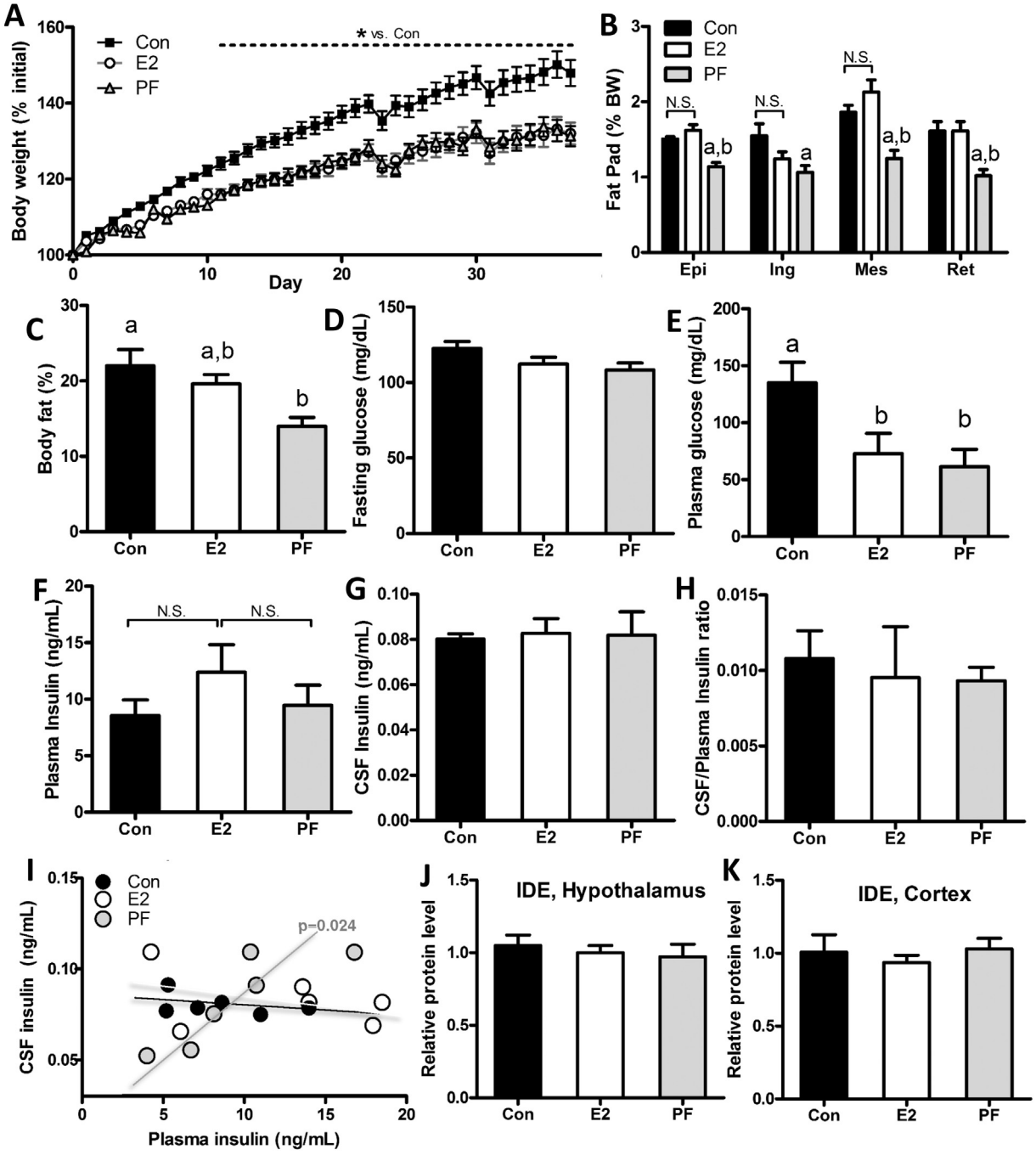

Fig. 5. E2 prevents HFD-DIO in male rats independently of the BBB-insulin-transport system. (A) 14-week-old HFD-DIO male rats were treated with E2 or vehicle with ad-lib access to HFD or were pair-fed to the body weight of E2-treated rats for 37 days. (B) Fat pad masses of the rats were expressed as percent body weight. Body fat percentage was significantly lower in PF, but not E2-treated rats (C). Fasting glucose levels were not different among groups (D) but 30 min after ip injection of 1 U/kg insulin, plasma glucose was significantly lower in E2-treated and PF rats compared to controls (E). Plasma insulin (F) and CSF insulin (G) were measured from samples collected 30-min following ip insulin (F). The ratio of CSF to plasma insulin did not differ among groups (H). CSF insulin levels were correlated with plasma insulin levels and the Pearson coefficient was calculated (I). The slope of the PF line was significantly different from zero ($P = 0.0135$, $r^2 = 0.7565$), whereas there was no significant correlation in the E2 group ($P = 0.4442$, $r^2 = 0.1524$) or controls ($P = 0.3741$, $r^2 = 0.1998$). The expression of insulin-degrading enzyme (IDE) in the hypothalamus (J) and cortex (K) was also assessed from brains of the same rats tested in this study. $n = 6–7$, $^*P <; 0.05$, and +/− SEM.

prior to insulin injection [10]. This baseline difference could potentially mask any differences of insulin transport into the CSF among groups, so we used an alternative approach that is effective in assessing insulin transport under these conditions [10]: linear regression analysis revealed that only PF rats had a significant Pearson correlation between the level of insulin in the blood and CSF following the prevention of HFD-DIO (Fig. 5I), indicating that a portion of the administered insulin was transported into the CNS. In contrast, E2-treated and control rats had no such correlation, implying that insulin transport into the CNS was not improved in these groups. Surprisingly, this suggests that in rats of the same body weight, E2 may actually inhibit the transport of insulin into the CNS when maintained on a HFD. It is also possible that E2's ability to prevent and reverse HFD-DIO and to protect peripheral insulin sensitivity occurs independently of changes in BBB-insulin transport. These possibilities raise a number of questions that warrant future study.

In comparison with the cohort of rats that underwent a reversal of HFD-DIO, Pearson analysis revealed a nonsignificant correlation for all

groups (Fig. 4H). It is interesting that insulin transport into the CNS was associated with improved insulin transport only when HFD was not consumed chronically prior to the start of treatments.

Because our approach measures unbound insulin in CSF samples, the detection of insulin depends not only on the rate of insulin transport into the CNS, but also on its subsequent degradation in the CNS and the relative amount of insulin that is bound to insulin receptors on brain cells. We did not observe a difference in the expression of insulin degrading enzyme in the brains of chronically E2-treated, PF, or control rats, implying that differential insulin degradation in the CNS is unlikely to be a factor. Additionally, obesity decreases the expression of insulin receptors at the BBB, which is thought to reduce insulin transport into the CNS [54]. E2-treatment does not affect insulin binding in the brains of OVX rats [32], making it is unlikely that insulin is transported differentially in E2-treated rats, and that the difference is masked by higher binding of insulin to brain cells. Taken together, these observations strengthen our conclusion that E2 neither alters the transport of insulin into the CNS nor its degradation therein.

Since systemic insulin sensitivity did not significantly differ between PF and E2-treated rats, it is intriguing to ask what other factors might be playing a role in the increased BBB-insulin transport of PF rats. Since total body fat and visceral fat are associated with impaired insulin transport into the CNS [10,11,18], we examined the relationship with adiposity to account for the differences noted in insulin transport between E2-treated and PF rats. Rats that underwent treatment for the reversal of HFD-DIO for 5 weeks (Fig. 4A) displayed clear changes in body weight, which corresponded with a lower proportion of epididymal fat in PF rats relative to E2-treated rats (although there was no change in the total body fat percentage), but this did not influence BBB-insulin transport, and this also did not appear to affect the metabolic syndrome to a substantial degree.

In contrast, in the follow-up study of HFD-DIO-prevention, pair-feeding led to a significantly lower percentage of total body fat and visceral fat storage relative to both E2-treated and control rats. The reduction of adiposity exclusively in PF rats might potentially explain why pair-feeding was associated with protected BBB-insulin transport. Further studies are necessary to determine the mechanism responsible for this difference. It is surprising that E2-treatment did not reduce the proportion of visceral fat storage and did not lead to increased subcutaneous fat in male rats, despite many publications reporting that E2 promotes increased storage of subcutaneous fat in a "gynoid" distribution [18,55]. However, several reports examining the effects of E2-treatment in male and OVX rats and mice have reported similar findings to ours [56,57]. It is possible that testosterone interacts with E2 to alter the typical fat distribution and/or that HFD alters the fat distribution normally maintained by E2. Regardless of differences in fat distribution, our results are consistent with previous findings that E2 increases adiposity relative to weight-matched controls (either OVX or males) [58,59].

The majority of insulin in the CNS is thought to be derived from the transport of blood-borne insulin through the BBB [45,60,61]. After entering the brain interstitial fluid compartment, insulin can bind its receptors on brain cells. The bulk flow of unbound insulin then makes its way into the CSF and is then transported back into the blood. Based on this model, the most ideal assessment of insulin transport into the CNS would involve measuring the insulin concentration in brain interstitial fluid before and after ip-insulin administration. Unfortunately, the technical demands of interstitial fluid collection limit its reliability. CSF collection is a strong alternative [62,63], because a large volume can be collected rapidly at high purity and the concentration of insulin in the CSF correlates strongly with the amount of insulin in the brain [36,64].

If insulin transport in the CNS were increased by E2, then females or E2-treated subjects might be expected to have higher concentrations of insulin in the CNS than males or vehicle-treated subjects of the same age and body weight. One study examining this question found that healthy women and men do not differ in the concentration of insulin in the CSF [65]. However, data on this matter are limited by a lack of consistency between individual CSF samples, such as not using a standardized fasting duration prior to CSF withdrawal, a factor known to affect the insulin concentration in the plasma and CSF [66]. The menopausal status of female subjects should also be accounted for when analyzing such data, to avoid differences in the levels of E2 in this group. Our data circumvent these issues by conducting the studies in a controlled manner with a standardized diet.

Taken together, our data demonstrate that E2 can improve energy balance and systemic insulin sensitivity independently of altering BBB-insulin transport. Further, our data indicate that food restriction may protect BBB-insulin transport during maintenance on a HFD. These results raise intriguing questions as to how E2 reduces insulin sensitivity in the brain and suggest the involvement of E2 in the modulation of neuronal or glial insulin signaling in the brain. Understanding this system could lead to the development of new therapeutic strategies in the prevention and treatment of obesity.

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