



Critical Role of the Endothelial α V/ β 5 Integrin Receptor Signaling Pathway in Promoting Irisin-induced Hippocampal BDNF Expression

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Abstract

Irisin, a myokine released into the bloodstream during physical exercise, is recognized for its role in modulating hippocampal brain-derived neurotrophic factor (BDNF) expression. However, the exact mechanisms by which irisin elevates BDNF levels are not fully understood. Notably, the α V/ β 5 integrin receptor, identified as a critical receptor for irisin primarily observed in bone and adipose tissues, was shown to play a key role in endothelial cells. Considering that irisin has nitric oxide (NO)-dependent vasodilatory effects and that NO increases endothelial BDNF production, with approximately 50% of cerebral BDNF expression derived from cerebral endothelium, we aimed to explore the molecular mechanisms through which irisin regulates BDNF levels in the hippocampus with a focus on the endothelium. Our findings indicated that recombinant irisin (r-irisin) treatment of hippocampal slices led to an increase in BDNF expression and that this effect was blocked by cilengitide, an α V/ β 5 integrin antagonist. Mechanistically, r-irisin activated focal adhesion kinase (FAK), and this activation was also inhibited by cilengitide. In vivo, we observed that exercised rats exhibited enhanced FAK activation with a positive correlation between FAK phosphorylation and serum irisin levels as well as FAK phosphorylation and hippocampal BDNF expression. Additionally, α V/ β 5 integrins, phosphorylated endothelial NO synthase (p-eNOS), and BDNF were significantly elevated in the hippocampal endothelial cells of exercised rats. These findings reveal that irisin may upregulate BDNF expression in the hippocampal through a mechanism dependent on endothelial α V/ β 5 integrins and the FAK/eNOS signaling pathway, supporting its potential as a therapeutic target for enhancing NO-dependent BDNF expression.

Keywords Irisin signaling · BDNF · α V/ β 5 integrin receptor · NO production · Hippocampus · Vascular endothelium

Introduction

Among the mechanisms contributing to the overexpression of brain-derived neurotrophic factor (BDNF) associated with neuroprotective effects across various brain regions, the humoral/endocrine pathway has recently been extensively highlighted. It is well-documented that myokines, which are

secreted into the bloodstream by skeletal muscles during contraction in response to exercise (EX), are linked to the overexpression of BDNF in the brain (for review [1, 2]). Identified among these myokines, irisin, which is generated from the cleavage of fibronectin type III domain-containing protein 5 (FNDC5) and released into the circulation [3], has been recognized as an important modulator of BDNF production within the brain [4], although the precise mechanisms of its action remain to be fully understood. Research indicates that peripheral delivery of FNDC5 by adeno-associated virus strategy is associated with elevated irisin plasma levels and an increase in *Bdnf* gene expression in the hippocampus (HP) [4]. Islam et al. [5] suggested that this myokine appears to cross the blood-brain barrier (BBB) and upregulates the expression of genes related to trophic factors in the brain, potentially contributing to the regulation of mood and anxiety [6, 7]. Other suggested roles of irisin include the enhancement of spatial learning and memory through long-term potentiation (LTP) [5, 8, 9], the improvement of neurological outcomes in rodent stroke

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models and Parkinson's diseases [10–12] and the reduction of neuroinflammation, which supports neuronal survival and function [11–14]. Although these multiple effects and data from Islam et al. (2021) suggested that irisin acts within the brain, it is important to emphasize that irisin also has an effect on cerebral vessels, as it has been shown to exert vasodilatory effects possibly through a nitric oxide (NO)-dependent mechanism [15, 16], which may partially account for its influence on cognition and neurological outcomes. Considering these studies, it is important to note that reported functional improvements have largely been confined to pathological contexts (e.g., hypertension, obesity), and it remains unresolved whether this axis confers measurable benefits in healthy brain vessels.

We recently demonstrated that peripheral irisin may mediate EX-induced BDNF expression independently of hippocampal FNDC5/irisin upregulation, suggesting that irisin could primarily signal through the cerebral endothelium to produce its beneficial effects [17]. Specifically, irisin may interact at the BBB through the integrin-like $\alpha V/\beta 5$ receptor, which has been identified as a critical receptor for irisin's action, mediating its signaling in various tissues, including bone and fat [18, 19]. Thus, this receptor is also expressed in endothelial cells [20–23] where its primary functions include the reduction of apoptosis and the enhancement of angiogenesis. These effects are mediated by the major downstream effector of the $\alpha V/\beta 5$ integrin receptor which is the focal adhesion kinase (FAK) [18–20, 24]. In addition, irisin was shown to enhance endothelial function by promoting the phosphorylation of endothelial nitric oxide synthase (eNOS) at serine 1177 (p-eNOS^{S1177}), which is crucial for NO production, this activation resulting in vasodilation and improved blood flow [25–27]. Moreover, eNOS-derived NO was shown to act as a positive transcriptional modulator of BDNF expression [15, 16, 28, 29].

Therefore, the aim of the present study was to investigate whether peripheral irisin could interact with the $\alpha V/\beta 5$ receptor located in cerebral microvessels and activate irisin-mediated signaling pathways to enhance BDNF expression in the HP. To this end, we designed *ex vivo* and *in vivo* experiments to investigate the effects of irisin on both FAK and eNOS activation in irisin-incubated hippocampal slices treated or not with the $\alpha V/\beta 5$ integrin antagonist, cilengitide, and in the HP of exercised rats with elevated serum irisin levels.

Materials and Methods

Animals

Experiments were carried out on 8-week-old male Wistar rats ($n = 38$) according to the French Department of

Agriculture guidelines (APAFIS #33300) and approved by the local ethics committee (C2EA, Dijon, n°105). Rats, purchased from Janvier Labs (Le Genest Saint Isle, France), were housed 5 per cage, kept under a 12/12 h light/dark cycle and allowed *ad libitum* access to food and water.

In vivo studies

Fourteen rats were allocated to the *in vivo* studies and underwent a 7-day habituation protocol to acclimate them to the experimenter and the treadmill apparatus.

EX protocol and animal groups

As previously described in Leger et al. (2024), after the habituation period, all animals were first subjected to an incremental EX test to exhaustion to determine their maximal aerobic speed (MAS). Refractory animals to the treadmill EX during the habituation period were excluded from the experiment ($n = 2$). Based on their MAS values, animals were assigned to two experimental groups: sedentary (SED, $n = 6$) and medium intensity EX (treadmill speed set at 14 m/min, 50% of MAS, EX, $n = 6$). Rats assigned to the EX group underwent treadmill EX for 30 min per day on a horizontal treadmill each morning for 7 consecutive days, while SED rats remained in their own cage in the vicinity of the treadmill apparatus.

Collection of samples

Twenty-four hours after the last treadmill session (day 15), all rats were anesthetized with a ketamine (75 mg/kg, Virbac, Carros, France)/xylazine (8 mg/kg, Bayer, Leverkusen, Germany) mix (0.1 mL/100 g, i.p.) following premedication with buprenorphine (0.05 mg/kg, s.c., Axience, Pantin, France) 30 min before anesthesia. A 5-min transcardiac saline perfusion was performed to flush out blood from the vasculature. After decapitation, the brain was extracted and HP of the right hemisphere was collected, weighed and promptly frozen at -80°C until protein extraction. Right HP samples were homogenized in 7 volumes of ice-cold lysis buffer [100 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM ethylene glycol tetraacetic acid, 1% triton 404 X-100, 1% protease inhibitor cocktail (P8340, Sigma-Aldrich, St-Quentin-Fallavier, France), 1% Halt™ phosphatase inhibitor cocktail (1862495, Thermoscientific, Illkirch-Graffenstaden, France)] and the protein concentration of supernatants was measured using the Lowry method (Modified Lowry Protein Assay kit, 23,240, Thermoscientific, Illkirch-Graffenstaden, France).

The left HP was immediately fixed in paraformaldehyde solution (4% PFA, 9713, VWR, Fontenay-sous-Bois, France) for 48 h, before being dehydrated using increasing ethanol

gradients, clearing with xylene and embedding in paraffin at the ImaFlow core facility (automaton ASP300, *Leica*, US58 BioSanD, Dijon, France). Paraffin-embedded HP sections were cut with a microtome (HM325, *Microm Microtech*, Brignais, France) in 5 µm thick cross-sections, spread on SuperFrost Plus™ (J1800AMNZ, *Epredia*, Neuilly-Sur-Seine, France) slides and dried overnight at 45 °C.

Ex Vivo Studies

Twenty-four rats were allocated to the ex vivo studies and underwent a 5-day habituation protocol to acclimate them to the experimenter.

Collection of Samples

All rats were anesthetized with a ketamine (75 mg/kg, Virbac, Carros, France)/xylazine (8 mg/kg, Bayer, Leverkusen, Germany) mix (0.1 mL/100 g, i.p.) following premedication with buprenorphine (0.05 mg/kg, s.c., Axience, Pantin, France) 30 min before anesthesia. A 5-min transcardial saline perfusion was performed to flush out blood from the vasculature. After decapitation, brains ($n = 24$) were extracted and all HP of the two hemispheres were collected and immediately pooled in a solution at 4 °C containing a culture medium (M199 medium supplemented with L-glutamine [L0361, *Dutscher*, Issy-les-Moulineaux, France], 33.5% fetal bovine serum [P190801, 500105G1G, *Dutscher*, Issy-les-Moulineaux, France], 2% D-glucose [346987-CER, *Dutscher*, Issy-les-Moulineaux, France], 2% amino acids [B6766, *Merck*, Saint-Quentin Fallavier, France] and 1% antibiotic antimycotic solution [A5955, *Merck*, Saint-Quentin Fallavier, France]).

Preparation and Incubation Conditions of Hippocampal Cultured Samples

Once collected in the culture medium and pooled to circumvent potential confounding effects due to differential responses among hippocampal regions, all HP were sliced (1 × 1 mm) using a McIlwain tissue chopper and allocated into wells of a 48-well plate (353078, *Dutscher*, Issy-les-Moulineaux, France) at the rate of 40 mg/mL. Then, slices were incubated in 9 experimental conditions (6 wells/condition) at 37 °C in a 95% O₂- 5% CO₂ atmosphere for 16 h: 1/CT (culture medium only), 2/Cil. 10 (cilengitide at 10 µM), 3/r-irisin 50 (recombinant irisin at 50 nM), 4/r-irisin 100 (recombinant irisin at 100 nM), 5/r-irisin 100 + Cil. 1, and 6/r-irisin 100 + Cil. 10. R-irisin was added to cilengitide 1 h after the start of incubation. After incubation, slices were then washed and homogenized for Western blotting analysis.

Protein Extraction

The hippocampal slices were removed from wells and homogenized in 7 volumes of ice-cold lysis buffer [100 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM ethylene glycol tetraacetic acid, 1% Triton X-100, 1% protease inhibitor cocktail (P8340, *Sigma-Aldrich*, St-Quentin-Fallavier, France), 1% Halt™ phosphatase inhibitor cocktail (1862495, *Thermoscientific*, Illkirch-Graffenstaden, France)]; the protein concentration of supernatants was measured using the Lowry method (DC Protein Assay Reagents Package, 5000116, *BioRad*, Marnes-la-Coquette, France).

Biochemical Analysis

Western Blotting

Equal amounts of proteins were loaded on a sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE, concentration gel 4% and separation gel 15%) or TGX Stain-Free Fast Cast Acrylamide gel (7.5%, 15-well gel, 1610181 *BioRad*, Marnes-la-Coquette, France) or Criterion™ TGX Stain-Free Precast gel (Any kDa, 26-well gel, 5678125 *BioRad*, Marnes-la-Coquette, France), separated and electrophoretically transferred to nitrocellulose membranes (0.2 µm, 1620112 *BioRad*, Marnes-la-Coquette, France) using the semi-liquid method (Turbo Transblot, 1,704,150, *BioRad*, Marnes-la-Coquette, France). After blocking non-specific binding sites with a 5% solution of nonfat dry milk in Tris-buffered saline (TBS, 20 mM Tris/HCl, 137 mM NaCl, pH 7.4) containing 0.1% Tween 20 (TBS-T), membranes were probed with different primary and secondary antibodies according to the target protein (Table 1). Protein–antibody complexes were visualized using the enhanced chemiluminescence Western blotting detection system ECL clarity (1705061 *BioRad*, Marnes-la-Coquette, France). The band densities were determined using the ChemiDoc Imaging System (*BioRad*, Marnes-la-Coquette, France) and analyzed using ImageLab software (version 6.1.0 build 7, Standard Edition, *BioRad*, Marnes-la-Coquette, France). Internal standard markers (total proteins) were used to normalize the band densities. The appropriate amounts of total proteins to be analyzed were determined from concentration (increasing amounts of proteins)/response (band density) curves from two rats both belonging to a particular group (on the same gel). The in vivo experimental SED and EX groups were analyzed on a 15-well gel only whereas the ex vivo conditions were compared on a 26-well or 15-well gel. Gels were run in duplicate with identical amounts of protein for each sample load in the gel. Data were analyzed from a representative membrane shown above each graph.

Table 1 Primary and secondary antibodies used for Western blotting

Target proteins, MW	Primary antibody			Secondary antibody	
	Dilution	Reference	Supplier	Host and clonality	
BDNF (mature form), 14 kDa	1/3000 + nonfat dry milk 5%	ab108319	Abcam	Rabbit monoclonal	Jackson ImmunoResearch Lab 111–035–144, anti-rabbit, HRP-conjugated goat polyclonal, 1/30000
FAK, 125 kDa	1/3000 + nonfat dry milk 5%	#3285	Cell Signaling Technology	Rabbit polyclonal	Jackson ImmunoResearch Lab 111–035–144, anti-rabbit, HRP-conjugated goat polyclonal, 1/30000
p-FAK ^{Y397} , 125 kDa	1/3000 + nonfat dry milk 5%	#3283	Cell Signaling Technology	Rabbit polyclonal	Jackson ImmunoResearch Lab 111–035–144, anti-rabbit, HRP-conjugated goat polyclonal, 1/30000
α V/ β 5 integrins, 121 kDa	1/3000 + bovin serum albumin 5%	bs-1356R	Bioss	Rabbit monoclonal	Jackson ImmunoResearch Lab 111–035–144, anti-rabbit, HRP-conjugated goat polyclonal, 1/30000
p-eNOS ^{S1177} , 133 kDa	1/2000 + bovin serum albumin 5%	#612393	BD Biosciences	Mouse monoclonal	Jackson ImmunoResearch Lab 115–035–166, anti-mouse, HRP-conjugated goat polyclonal, 1/30000

Immunofluorescence

Brain slices were deparaffinized and rehydrated in various successive baths of xylene and ethanol, then washed in Tris-buffered saline (TBS, T6664, *Sigma-Aldrich*, St-Quentin-Fallavier, France). Epitope retrieval was then induced by heating slices (95 °C for 20 min) in Sodium Citrate buffer (Trisodium citrate, 10 mM, 0.1% Tween 20, pH 6.0) bath. After blockade of nonspecific binding sites with TBS containing 5% of goat serum (GS), 0.1% of triton X100, 0.2% tween20 for 30 min at room temperature (RT), slides were washed with TBS and then incubated overnight at +4 °C with specific primary antibody in TBS with 2% of GS, 0.1% of triton X100, 0.2% tween20: an anti- α V/ β 5 antibody [bs-1356R, *Bioss* (Nanterre, France), rabbit polyclonal, 1/100], an anti-p-eNOS antibody [GTX129058, *GeneTex* (*CliniSciences*, Nanterre, France), rabbit polyclonal, 1/100], an anti-BDNF antibody [ab108319, *Abcam* (Cambridge, UK), rabbit monoclonal, 1/100] and an anti-Glut1 antibody [MABS132, *Sigma-Aldrich* (St-Quentin-Fallavier, France) mouse monoclonal, 1/100]. Every immunofluorescence was realized with a negative control, i.e. slice without primary antibody, to visualize eventual nonspecific background staining and tissue autofluorescence. After TBS washing, hippocampal slides were incubated for 60 min with fluorescent secondary antibodies: Alexa Fluor –568 or –488 in TBS containing 2% of GS, 0.1% of triton X100, 0.2% tween20. Finally, brain sections were mounted between slide and coverslip using Fluoro-Gel mounting medium with DAPI (FP-DT094A, *Interchim*,

Montluçon, France). Slides were observed and acquired by using an epifluorescence microscope (Axioscop Imager.M2, *Zeiss*, Oberkochen, Germany). Quantitative analysis of staining was conducted in duplicate using ZEN 2 software (blue edition, v3.7; *Zeiss*) by observers blinded to experimental groups. For all immunofluorescence analyses (α V/ β 5, p-eNOS, BDNF), fluorescence intensity was quantified exclusively within GLUT1-positive endothelial regions among the 5–7 microvessels present in each slide. GLUT1-positive structures were first identified in the GLUT1 channel, and regions of interest (ROIs) were manually delineated to follow endothelial profiles while excluding nuclei. Mean fluorescence intensity of the target marker was then measured within these ROIs.

Data and Statistical Analysis

GraphPad Prism 9.5.0 (Dotmatics, Boston, MA, USA) was used for statistical analysis and graph creation. Data were expressed as means $\pm$ standard deviations (SD). Based on normality and equal variance (Shapiro–Wilk test), differences were assessed using (1) a parametric *t*-test or non-parametric Mann–Whitney test for two groups, (2) an ordinary one-way ANOVA (Fisher's LSD or uncorrected Dunn's test) or Kruskal–Wallis test for three groups, and (3) a Pearson (r_p) or Spearman (r_s) test (unpaired values) for correlations. A value of $p < 0.05$ was considered statistically significant. The Dixon test identified extreme values as outliers, allowing their exclusion from statistical analyses.

As previously published in Leger et al. [17], in vivo BDNF protein expression data were illustrated only as a correlation between p-eNOS and BDNF protein levels.

Results

Effect of Irisin on Hippocampal BDNF Expression in the Presence or Absence of an α V/ β 5 Receptor Antagonist

Our primary objective was to evaluate the dose-dependent effects of irisin on hippocampal BDNF expression. To this end, hippocampal slices were incubated with two concentrations of recombinant irisin (r-irisin; 50 and 100 nM). Western blotting analysis revealed that r-irisin treatment significantly increases hippocampal BDNF levels ($+127.8 \pm 32.0\%$, $p < 0.01$ for 50 nM and $+172.8 \pm 105.1\%$, $p < 0.001$ for 100 nM) compared to the standard control condition (CT) (Fig. 1a).

Given that the 100 nM dose of r-irisin induces BDNF overexpression comparable to that observed after an exercise session at 50% of MAS, which also significantly increases circulating irisin levels [17], we selected this higher dose for subsequent experiments.

In the following experiments, hippocampal slices were incubated with 1 μ M or 10 μ M cilengitide (Cil.), an antagonist targeting the α V/ β 5 integrin receptor, in the presence of 100 nM

r-irisin. Our data showed that Cil. incubation inhibited the irisin-induced increase in BDNF levels, with the most pronounced reduction observed at the highest concentration: ($-34.5 \pm 11.2\%$, $p < 0.01$ for 1 μ M and $-57.6 \pm 13.3\%$, $p < 0.001$ for 10 μ M Cil. compared to 100 nM r-irisin, Fig. 1b). Based on this result, we selected the 10 μ M concentration for subsequent experiments.

To confirm the specificity of Cil.'s effect on BDNF expression in hippocampal slices, we first compared BDNF levels between CT conditions and treatment with 10 μ M Cil. alone (Table S1). As no significant difference was detected between these conditions, the 10 μ M Cil. condition was adopted as the new baseline control for all subsequent experiments. Under these conditions, treatment with 100 nM r-irisin significantly increased BDNF levels, consistent with the effect observed in Fig. 1a. In contrast, co-incubation with 10 μ M Cil. and 100 nM r-irisin abolished this increase ($262.1 \pm 79.0\%$, $p < 0.001$ for 100 nM r-irisin and $110.8 \pm 68.0\%$, ns for 10 μ M Cil. + 100 nM r-irisin, compared to 10 μ M Cil., Fig. 1c).

Irisin enhances hippocampal BDNF expression via the FAK signaling pathway

To investigate the signaling effect of irisin binding to its α V/ β 5 integrin receptor, we assessed FAK activation by Western blotting in both ex vivo hippocampal slices incubated with r-irisin, in the presence or absence of Cil. (Fig. 2, left side), and hippocampal samples from exercised (EX) rats at 50%

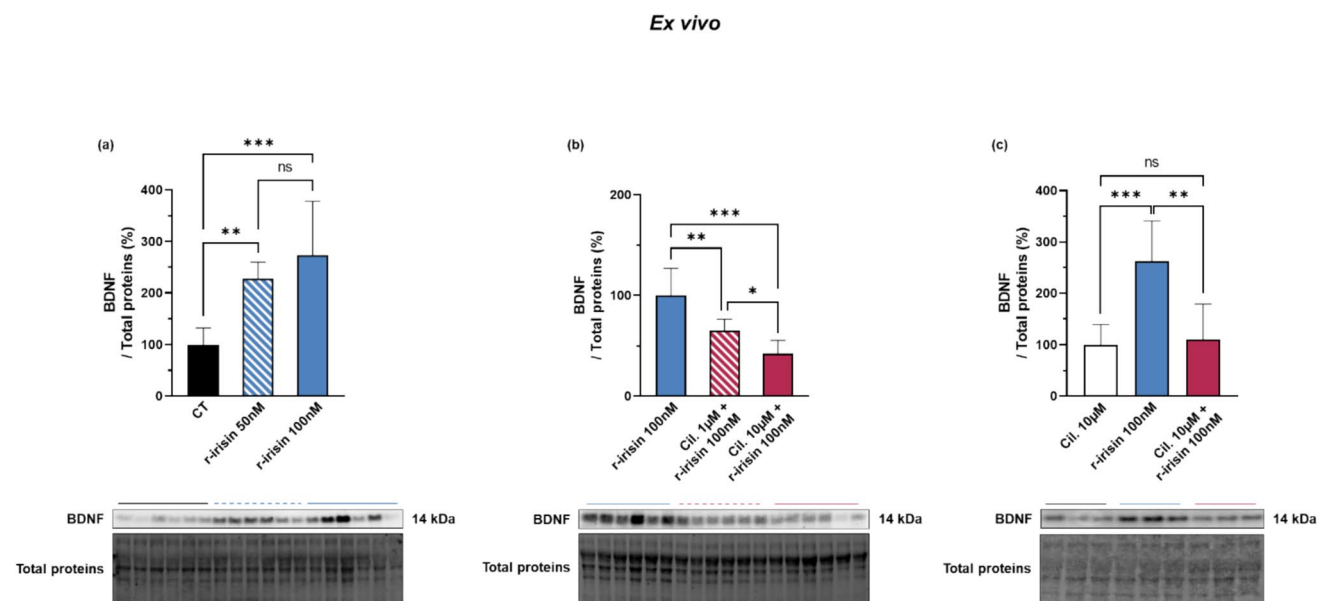


Fig. 1 Western blotting analysis of hippocampal BDNF expression in ex vivo experiments ($n = 6$ per condition). **a** Dose-dependent effect of r-irisin on BDNF expression. **b** Dose-dependent effect of cilengitide (Cil.) in the presence of 100 nM r-irisin. **c** Comparison of BDNF expression across selected experimental conditions. Values are

expressed as mean $\pm$ SD (%), normalized to: **a** CT, **b** 100 nM r-irisin and **c** 10 μ M Cil. conditions. Representative immunoblots are shown below each corresponding bar graph. ns: non-significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

MAS (Fig. 2, right side). In the ex vivo experiments, r-irisin induced phosphorylation of FAK ($191.7 \pm 47.9\%$, $p < 0.01$ compared to $10 \mu\text{M}$ Cil.), an effect that was abolished by co-treatment with Cil. ($94.1 \pm 54.4\%$, $p < 0.01$, compared to $10 \mu\text{M}$ Cil., Fig. 2a). Similarly, in the in vivo experiment, FAK phosphorylation was significantly increased in the EX relative to the SED group ($+70.4 \pm 38.7\%$, $p < 0.05$, Fig. 2c).

To assess whether FAK activation is associated with BDNF expression, we performed correlation analyses between p-FAK^{Y397} and BDNF levels under both experimental conditions. In both ex vivo and in vivo settings, p-FAK^{Y397} levels were positively correlated with BDNF expression (Fig. 2b, d). Furthermore, given our previous findings of elevated serum irisin levels in the EX compared to the SED group [17], we investigated the relationship between hippocampal p-FAK^{Y397} and serum irisin levels in the in vivo condition. A significant positive correlation was also observed between these variables (Fig. 2e).

Cellular localization and expression of the $\alpha\text{V}/\beta 5$ integrin complex

Given that the in vivo experiments demonstrated activation of the FAK signaling pathway following EX, we

next investigated the cellular localization of the $\alpha\text{V}/\beta 5$ integrin complex in brain sections passing through the HP, as well as its hippocampal expression in SED and EX rats ($n = 6$ per group). Since $\alpha\text{V}/\beta 5$ integrins are known to be expressed in endothelial cells, we used GLUT1 as an endothelial marker to assess whether the integrin complex was localized in the vascular endothelium. Our data confirmed the presence of $\alpha\text{V}/\beta 5$ integrins in the endothelium of hippocampal arterioles (Fig. 3a). Notably, endothelial staining intensity was significantly higher in EX compared to SED rats (Fig. 3b) with $\alpha\text{V}/\beta 5$ integrin expression nearly doubling in EX animals ($+85.2 \pm 28.7\%$, $p < 0.01$). While $\alpha\text{V}/\beta 5$ integrins were also detected in hippocampal neurons, qualitative inspection revealed no apparent difference in neuronal expression between the two groups.

To further characterize $\alpha\text{V}/\beta 5$ integrin expression, Western blotting analysis was performed on whole hippocampal tissue. Consistent with immunohistochemical findings, EX rats exhibited significantly higher expression of the $\alpha\text{V}/\beta 5$ integrin complex compared to SED ($+45.2 \pm 38.3\%$, $p < 0.05$, Fig. 3c). These results suggest that the expression of the $\alpha\text{V}/\beta 5$ integrin receptor is increased in cerebral endothelial cells in response to EX.

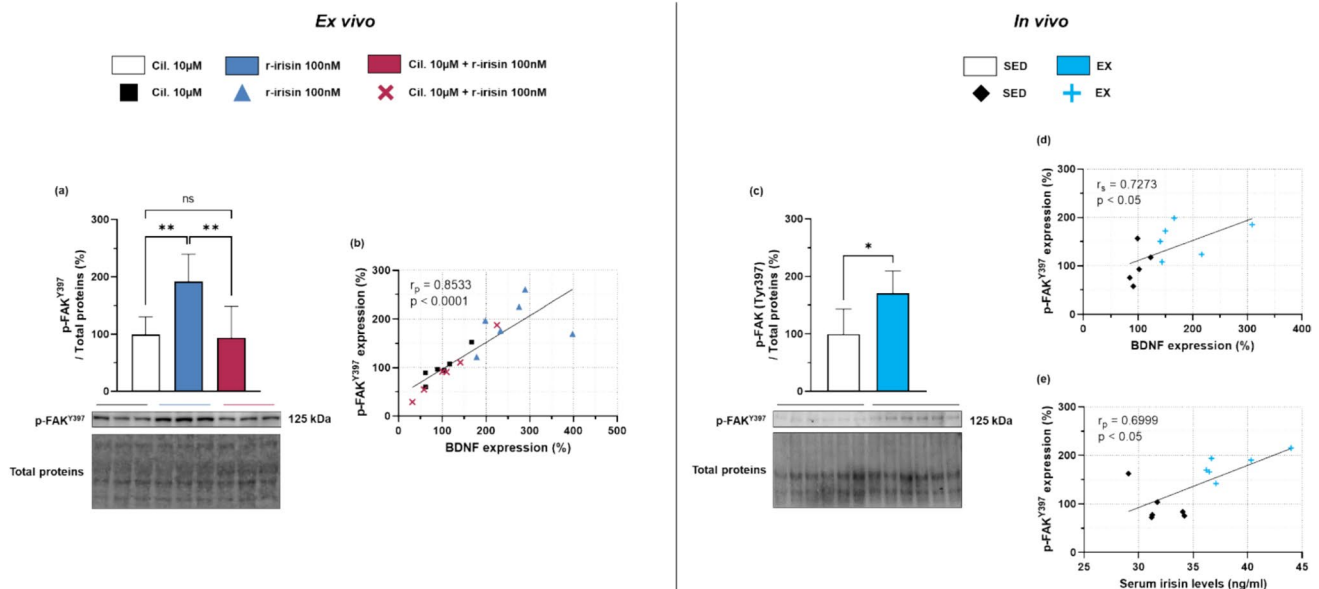


Fig. 2 Western blotting analysis of p-FAK^{Y397} expression and correlation with BDNF and serum irisin in ex vivo and in vivo ($n = 6$ per condition/group) experiments. **a** p-FAK^{Y397} expression in ex vivo hippocampal slices treated with $100 \mu\text{M}$ r-irisin, in the presence or absence of $10 \mu\text{M}$ cilengitide (Cil.), and corresponding immunoblots. **b** Correlation between p-FAK^{Y397} and BDNF expressions in ex vivo samples. **c** p-FAK^{Y397} expression in in vivo hippocampal samples

from sedentary and exercised groups, with corresponding immunoblots. **d** Correlation between p-FAK^{Y397} and BDNF expressions in in vivo samples, and **e** correlation between p-FAK^{Y397} expression and serum irisin levels in in vivo samples. Values are expressed as mean $\pm$ SD (% of $10 \mu\text{M}$ Cil. or SED group). ns: non-significant, * $p < 0.05$, ** $p < 0.01$

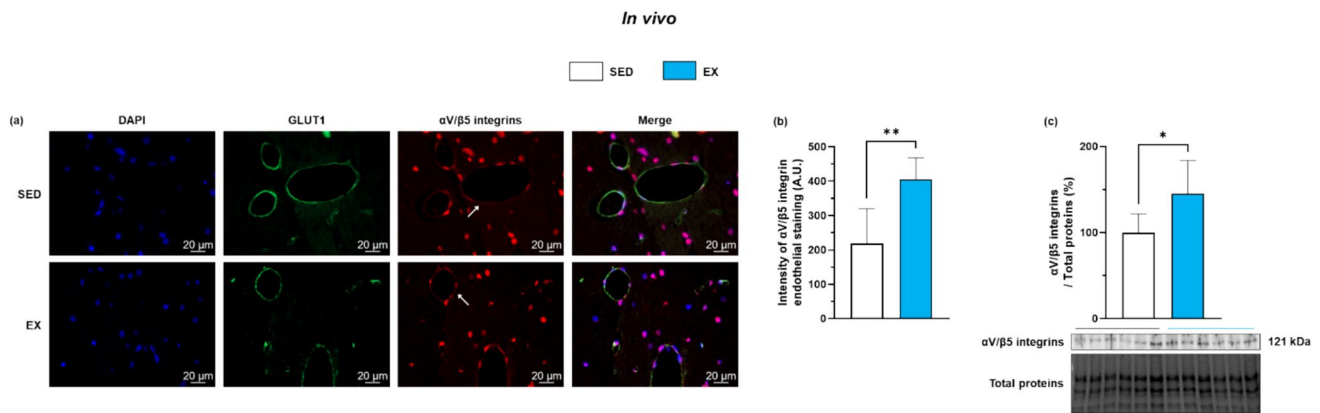


Fig. 3 Localization and expression of α V/ β 5 integrin receptor in the hippocampus of sedentary and exercised rats. **a** Representative α V/ β 5 integrin staining in hippocampal endothelial cells, co-labeled with the endothelial marker GLUT1, used to confirm endothelial identity, **b** quantification of α V/ β 5 integrin mean fluorescence intensity in

GLUT1-positive endothelial cells, and **c** Western blotting analysis of α V/ β 5 integrin complex expression in whole hippocampal tissue with corresponding immunoblot. Values are expressed as mean $\pm$ SD (A.U. or % of SED group). * $p < 0.05$, ** $p < 0.01$

Irisin enhances eNOS activation

Considering the known vasodilatory effect of irisin mediated by NO and given that BDNF synthesis is dependent on eNOS activation [30], we investigated whether irisin modulates eNOS activation in relation to BDNF upregulation. P-eNOS expression was first assessed by Western blotting in both ex vivo and in vivo experiments (Fig. 4a, d). In ex vivo conditions, treatment with r-irisin (100 nM) significantly increased p-eNOS expression compared to Cil. condition ($+107.3 \pm 40.9\%$, $p < 0.001$). This effect was abolished when r-irisin was added to incubation with Cil. In the in vivo experiment (Fig. 4d), EX performed at 50% of MAS significantly enhanced eNOS activation compared to SED animals ($+63.6 \pm 40.0\%$, $p < 0.05$). Importantly, p-eNOS expression was positively correlated with BDNF levels in both ex vivo ($r_p = 0.7133$, $p < 0.001$) and in vivo conditions ($r_s = 0.6636$, $p < 0.05$), (Fig. 4b, e).

Given that FAK activation was observed in both ex vivo and in vivo experiments, we examined its potential association with eNOS activation by assessing the correlation between p-FAK and p-eNOS expression levels (Fig. 4c, f). A significant positive correlation was found in both experimental contexts: ex vivo ($r_p = 0.7775$, $p < 0.001$) and in vivo ($r_s = 0.6273$, $p < 0.05$).

Cellular Localization and Expression of p-eNOS^{S1177} and Its Correlation with Endothelial α V/ β 5 Integrin and BDNF Expressions

To investigate the potential relationship between the α V/ β 5 integrin complex, NO signaling, and endothelial BDNF expression, we examined p-eNOS^{S1177} and BDNF expression in hippocampal slices from SED and EX groups

($n = 6$ per group). Immunofluorescence analysis showed that p-eNOS^{S1177} colocalized with the endothelial marker GLUT1 in hippocampal arterioles (Fig. 5a). Quantitative analysis showed a significant increase in endothelial p-eNOS expression in the EX group ($+48.7 \pm 29.3\%$ $p < 0.01$, Fig. 5b) compared to the SED group. Similarly, BDNF expression was localized to GLUT1-positive endothelial cells (Fig. 5c), and levels were significantly higher in the EX group ($+102.1 \pm 23.8\%$, $p < 0.01$, Fig. 5d) relative to SED animals.

Furthermore, in HP, positive correlations were identified between p-eNOS and BDNF expressions ($r_s = 0.6545$, $p < 0.05$, Fig. 5e), between endothelial α V/ β 5 integrin and p-eNOS expressions ($r_s = 0.7063$, $p < 0.05$, Fig. 5f), and between endothelial α V/ β 5 integrin and BDNF expressions ($r_s = 0.7909$, $p < 0.01$, Fig. 5g). These associations suggest that irisin-mediated effects on endothelial BDNF expression are at least partly driven by NO production.

Discussion

Given irisin's critical role in brain function, particularly its involvement in neuroprotection, neurogenesis, and cognition via modulation of multiple molecular pathways, early research on FNDC5/irisin primarily focused on neuronal cells. For instance, transduction of an FNDC5-expressing adenovirus into primary cortical neurons increased extracellular irisin levels and upregulated *Bdnf* gene expression [4]. In the same study, peripheral FNDC5 delivery to the liver via adenoviral vectors elevated circulating irisin, increasing hippocampal expression of *Bdnf* and other neuroprotective genes. Similarly, Huang et al. [31] showed, in diabetic rats, that manipulating irisin expression, either by overexpression

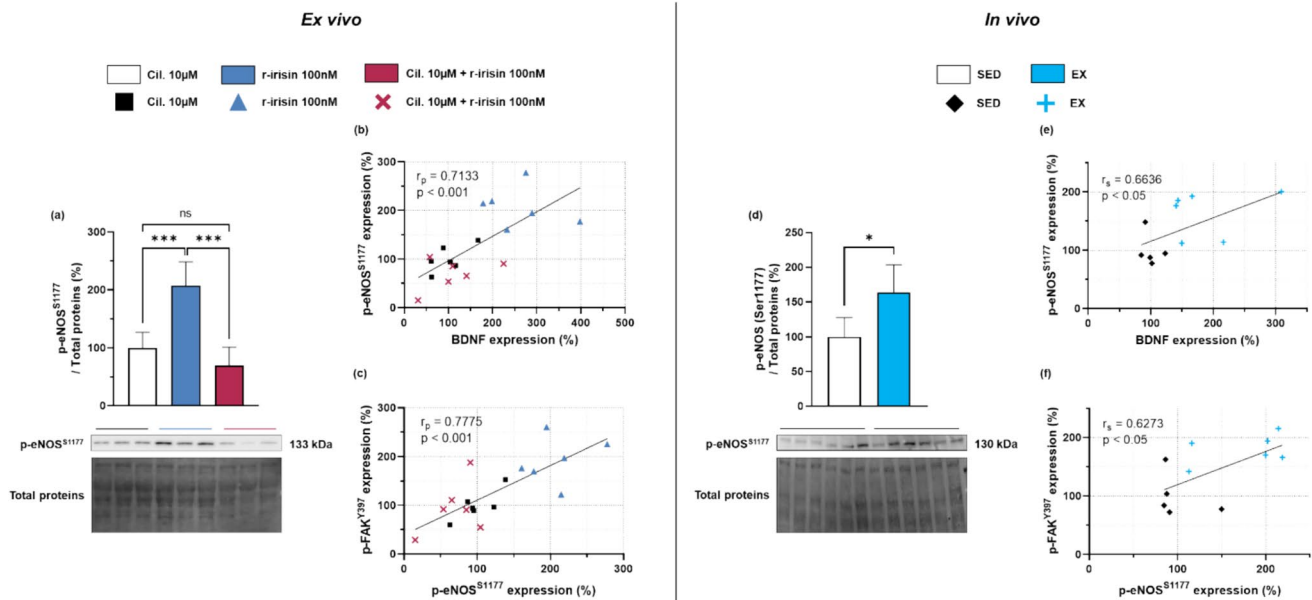


Fig. 4 Western blotting analysis of p-eNOS^{S1177} expression and correlations with BDNF and p-FAK^{Y397} in ex vivo and in vivo ($n=6$ per condition/group) experiments. **a** Expression of p-eNOS^{S1177} in ex vivo hippocampal tissue, with corresponding immunoblot, **b** correlation between p-eNOS^{S1177} and BDNF expressions in ex vivo conditions, **c** correlation between p-eNOS^{S1177} and p-FAK^{Y397} expressions in ex vivo conditions, **d** expression of p-eNOS^{S1177} expression

in in vivo hippocampal samples from sedentary and exercised groups, with corresponding immunoblot, **e** correlation between p-eNOS^{S1177} and BDNF expressions in in vivo samples, and **f** correlation between p-eNOS^{S1177} and p-FAK^{Y397} expressions in in vivo samples. Values are expressed as mean $\pm$ SD (% of 10 μ M Cil. or SED group). ns: non-significant, * $p < 0.05$, *** $p < 0.001$

or RNA interference, resulted in corresponding changes in hippocampal BDNF levels. Further supporting a peripheral mechanism, Lourenco et al. [8] demonstrated that intraperitoneal administration of anti-FNDC5 antibodies in exercised Alzheimer's disease (AD) mice reduced hippocampal FNDC5/irisin levels, confirming its role in EX-induced neuroplasticity. Our findings, that EX increases both circulating irisin and hippocampal BDNF, and that irisin incubation induces BDNF upregulation in hippocampal extracts, align with these prior observations. Building on studies suggesting that EX-induced BDNF expression in the HP is primarily driven by peripheral rather than central FNDC5/irisin [8, 17], we aimed to investigate whether irisin exerts part of its cerebral effects by engaging signaling pathway within the cerebral endothelium.

The mechanisms by which irisin acts in the brain to induce BDNF expression remain incompletely understood. Spiegelman's group proposed that irisin crosses the BBB and binds to α V/ β 5 integrin receptor in the brain, thereby enhancing cognitive function in mouse models of AD [5]. The involvement of α V/ β 5 integrins as putative irisin receptors was further supported in a model of intracerebral hemorrhage. Specifically, administration of cilengitide, a cyclic pentapeptide containing the Arg-Gly-Asp (RGD) motif essential for integrin binding, selectively inhibited α V/ β 3 and α V/ β 5 integrins and abolished

irisin's neuroprotective effects, including its regulation of neuroinflammation and neuronal apoptosis [11]. Likewise, irisin-dependent activation of FAK via α V/ β 5 integrins was demonstrated in SH-SY5Y cells treated with irisin. In this context, blockade of α V/ β 5 by the internalizing RGD (iRGD) peptide abolished irisin-induced FAK phosphorylation [12]. Notably, this study was conducted in a context of Parkinson's disease model focused on neuronal integrin expression, despite known expression of these receptors in multiple brain cell types, including endothelial cells [20–23]. To further explore the molecular mechanisms by which irisin regulates BDNF expression, we incubated hippocampal slices with r-irisin, either alone or in combination with cilengitide. Our data are consistent with previous findings implicating integrin signaling: r-irisin treatment significantly increased hippocampal BDNF protein levels, an effect that was dose-dependently reversed by cilengitide co-application. We then examined downstream signaling and found that irisin enhanced FAK phosphorylation, an effect similarly abolished when cilengitide was co-applied with r-irisin. Ex vivo, we observed a positive correlation between FAK activation and BDNF expression, as p-FAK levels were significantly associated with BDNF protein expression. These findings, together with our in vivo data showing that EX induced hippocampal FAK activation as well as a significant positive correlation between p-FAK

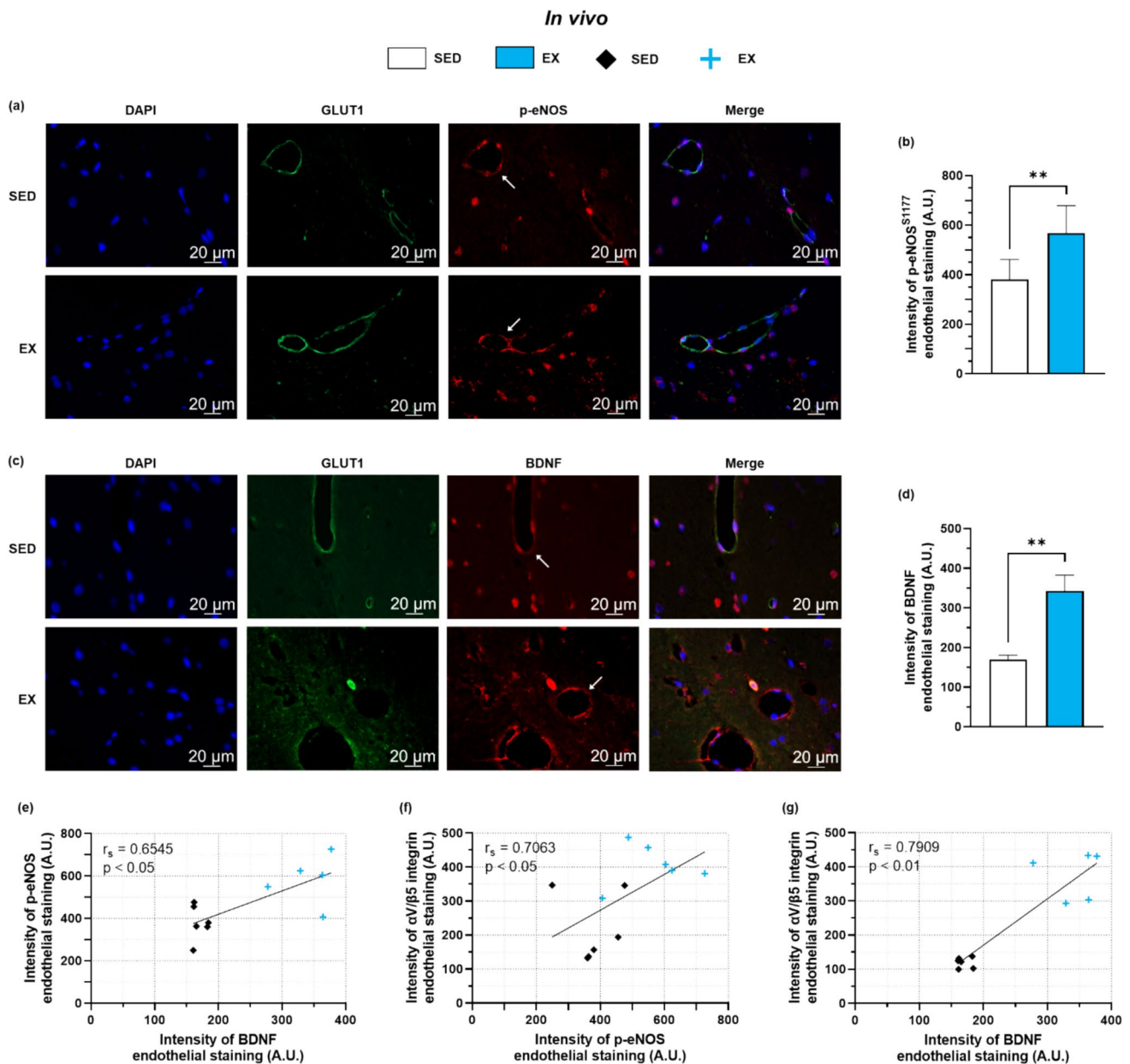


Fig. 5 Cellular localizations and expressions of p-eNOS^{S1177} and BDNF in the hippocampus, and correlations among endothelial p-eNOS, BDNF, and α V/ β 5 integrin expressions in sedentary and exercised rats. **a** Representative immunofluorescence staining showing p-eNOS^{S1177} colocalized with the endothelial marker GLUT1, used to confirm endothelial identity, in hippocampal arterioles, **b** quantification of the mean intensity of the p-eNOS^{S1177} staining in endothelial cells, **c** representative immunofluorescence stain-

ing showing BDNF colocalized with GLUT1, **d** quantification of the mean intensity of the BDNF staining in endothelial cells, **e** correlation between p-eNOS^{S1177} and BDNF expressions in hippocampal endothelial cells, **f** correlation between α V/ β 5 integrin and p-eNOS^{S1177} expressions in hippocampal endothelial cells, and **g** Correlation between α V/ β 5 integrin and BDNF expressions in hippocampal endothelial cells. Values are expressed as mean $\pm$ SD (A.U.). ** $p < 0.01$. Scale bars: 20 μ m

and serum irisin levels, support the involvement of α V/ β 5 activation and downstream FAK signaling in irisin-induced BDNF upregulation in the HP.

To further clarify the cellular context, we next examined the cellular localization of α V/ β 5 integrin expression. Notably, the α V/ β 5 integrin receptor has been primarily identified

in osteocytes and adipocytes [18, 19]. In the brain, Tang et al. [32] proposed that FNDC5 promotes BDNF expression in hippocampal neurons by binding to the neuronal α V/ β 5 integrin receptor, thereby enhancing synaptic plasticity. Other studies have shown that these integrins also mediate interactions with proteins involved in microglial adhesion

[33]. However, these investigations largely overlooked cerebral vasculature. As stated above, other reports have indicated that $\alpha V/\beta 5$ expression in the brain is predominantly localized to glial and endothelial cells rather than neurons [5, 34]. Indeed, $\alpha V/\beta 5$ integrins play a key role in the cardiovascular system, particularly in endothelial cells, where they regulate functions such as endothelial phenotype and vascular remodeling [35, 36]. Consistent with this, our findings demonstrated that the $\alpha V/\beta 5$ integrin receptor is expressed not only in neurons but also in endothelial cells of hippocampal microvessels in both SED and EX animals. Furthermore, EX-induced increases in $\alpha V/\beta 5$ expression were attributed to enhanced endothelial, rather than neuronal staining, as revealed by our immunohistochemical analysis. Increased hippocampal endothelial $\alpha V/\beta 5$ after exercise likely reflects shear stress-driven mechanotransduction at endothelial-ECM adhesions, consistent with the role of αV -containing integrins in flow-responsive signaling that supports vascular function and neurovascular integrity [37–40]. These results suggest that irisin may exert part of its effects via $\alpha V/\beta 5$ receptors at the endothelial level. This interpretation aligns with prior evidence of irisin's vasodilatory effects across different concentrations and underscores the potential role of this myokine in regulating vascular tone [15, 27, 41].

Given irisin's vascular effects, we investigated whether activation of the $\alpha V/\beta 5$ -FAK pathway is linked to eNOS activation as a potential mechanism contributing to BDNF expression. Indeed, previous studies have shown that NO positively regulates endothelial BDNF synthesis [30] and that nearly 50% of cerebral BDNF originates from endothelial cells [29] and contributes to cognitive function [42] as reported also in studies showing that eNOS KO mice exhibited cognitive impairments as compared to wildtype in different contexts of ischemic conditions [43–45]. Recent data indicate that endothelial-derived BDNF sustains local neuronal plasticity via an endothelial TrkB/NO axis—independently of neuronal BDNF—consistent with the anxiodepressive phenotype, memory deficits, reduced synaptogenesis, and lower hippocampal BDNF in BDNF^{ECKO} mice [42]. This endothelium-to-neuron signaling weakens in diabetes and aging (hyperglycemia, endothelial dysfunction, “inflammaging”), dampening BDNF/TrkB signaling, thereby compromising plasticity and highlighting endothelial BDNF as a potential biomarker and therapeutic target. In addition to this link between eNOS and BDNF, evidence indicates that FAK activation enhances eNOS phosphorylation, thereby promoting NO production [46, 47]. Although correlation does not imply causation, we observed a positive association between $\alpha V/\beta 5$ expression, p-FAK activation, and p-eNOS levels, suggesting a potential mechanistic link between integrin signaling and eNOS activation in vivo. Notably, FAK activation may also result from increased shear stress

induced by EX [46]. However, our ex vivo data strengthen the hypothesis of a direct irisin effect since incubation with r-irisin significantly increased eNOS activation, as indicated by elevated levels of p-eNOS. This effect was abolished by co-incubation with cilengitide, supporting the involvement of an integrin/FAK-dependent mechanism. Finally, endothelial BDNF staining was also increased in exercised animals, and the intensity of endothelial p-eNOS staining positively correlated with that of endothelial BDNF staining, while a similar association was observed between p-eNOS and BDNF expressions in both ex vivo and in vivo conditions, further supporting a role for the endothelial eNOS/NO pathway in irisin-induced BDNF upregulation.

Limitations should be considered when interpreting our findings. First, the use of cilengitide in our ex vivo experiments represents a limitation due to its partial specificity. While cilengitide is an antagonist of $\alpha V/\beta 5$ integrins, it also inhibits $\alpha V/\beta 3$ integrins, which are expressed in endothelial cells [48, 49]. The role of $\alpha V/\beta 3$ in the brain remains incompletely understood, although some studies suggest it may exert anti-inflammatory effects in neuropathological contexts such as cerebral ischemia [50]. Future work should employ more selective $\alpha V/\beta 3$ and $\alpha V/\beta 5$ antagonists or a targeted gene-silencing approach (e.g., siRNA) to dissect the contribution of each heterodimer. Second, we did not fully delineate the downstream mechanisms linking FAK activation to eNOS phosphorylation at the endothelial level. Based on the existing literature, several pathways, including AMPK/Akt [11, 15, 51], PI3K/Akt [12, 13, 15] and ERK1/2 [12, 52], could be involved in mediating this effect in cerebral endothelial cells. To move from correlation to mechanisms and establish causality within the irisin/FAK/eNOS pathway, further investigations using better endothelial-specific models such as microvessel-enriched fractions obtained from a large cohort of both male and female animals will be required to dissect the specific molecular intermediates involved. Should the ex vivo approach demonstrate molecular efficacy, confirmation in vivo—together with behavioral assays—will be required.

In conclusion, while our data do not exclude the possibility that irisin crosses the BBB and induces neuronal BDNF expression, they suggest an alternative or complementary mechanism. Specifically, our findings suggest that EX-induced BDNF overexpression may, at least in part, result from irisin acting on the cerebral endothelium, thereby enhancing endothelial BDNF expression through the $\alpha V/\beta 5$ -FAK-eNOS signaling pathway.

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Data Availability The datasets generated during and/or analysed during the current study are available on request from the corresponding author Anne Prigent-Tessier.

Declarations

Ethic Approval All experiments and protocols were approved by the French Department of regulated research practice (APAFIS numbers #33300–2021093014586596 and #28240–202011101408485) and the Ethics Committee in animal experimentation of Grand campus Dijon (agreement number 105, user establishment approval number E2146404EA). They complied with ARRIVE guidelines and conformed to the European convention for the protection of vertebrate animals used for experimental and other scientific purposes.

Competing Interests The authors declare no competing interests.

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