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Nutrigenomic modification induced by anthocyanin-rich bilberry extract in the hippocampus of ApoE-/- mice

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ABSTRACT

Dietary anthocyanins may slow cognitive decline, improve cognitive performance and exert neuroprotective effects against neurodegenerative disorders. However, the underlying mechanisms of their action are not fully understood. This study investigated the effects of 12-week anthocyanin-rich bilberry extract supplementation (0.02%) on global gene expression in the hippocampus of ApoE-/- mice to help the understanding of molecular mechanisms underlying anthocyanin neuroprotective effects. Gene expression analysis identified 1698 differently expressed genes, with 611 downregulated and 1087 upregulated genes. Bioinformatics revealed that these genes regulate different biological processes, including neurogenesis, inflammation, metabolism, cell to cell adhesion, cytoskeleton organization, and Alzheimer's and Parkinson's disease pathology. The bioinformatic analysis also proposed potential miRNAs and transcription factors that could be involved in the mediation of these nutrigenomic effects. Results from molecular docking also suggested that anthocyanins could bind to top transcription factors with, as potential consequence, an impact on their gene expression regulation. Taken together, integrated analysis revealed a multi-target mode of action of anthocyanin-rich bilberry extract in the hippocampus underlying their neuroprotective properties.

1. Introduction

Polyphenols are the largest group of phytochemicals widely found in plant foods such as fruits, vegetables, and plant-derived beverages. Over the past two decades, these compounds received much attention due to the diverse health-promoting effects associated with the consumption of polyphenol-rich foods. Indeed, various epidemiological, clinical, and preclinical studies support the role of polyphenols in the prevention and amelioration of different chronic diseases such as cardiovascular diseases (Kim & Je, 2017; Luís, Domingues, & Pereira, 2018; Menezes et al., 2017; Rodriguez-Mateos et al., 2019), type-2 diabetes (Cao et al., 2019), and neurodegenerative disorders (Lefèvre-Arbogast et al., 2018; Socci, Tempesta, Desideri, De Gennaro, & Ferrara, 2017). However, the exact molecular mechanisms underlying their health effects remain unclear.

One of the most studied polyphenols are anthocyanins, plant pigments present in berries and berry-derived products that exert a variety of biological activities, including different beneficial effects on brain function. In recent years, the effects of anthocyanin-rich foods or anthocyanins on cognition have been a focus of a growing number of studies. Data from epidemiological studies have shown that higher intakes of anthocyanin-rich berries are strongly associated with a slower cognitive decline in aged women and a lower probability of moderate and poor late-life cognitive function in men (Devore, Kang, Breteler, & Grodstein, 2012; Yuan et al., 2019). In a pooled analysis of two US cohort studies which examined almost 150 000 people, lower Parkinson's disease (PD) risk was associated with the highest quintile of anthocyanin intake (Gao, Cassidy, Schwarzschild, Rimm, & Ascherio, 2012). Clinical trials suggest that both acute and long-term consumption of anthocyanin-rich berries could improve some aspects of cognitive function across different age groups (Bensalem et al., 2019; Hein,

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Whyte, Wood, Rodriguez-Mateos, & Williams, 2019; Travica et al., 2019). Furthermore, favorable changes in cognitive performance (e.g., spatial and short-term memory) have been reported in animal models following dietary supplementations with anthocyanin-rich berry extracts or pure anthocyanins (Khan M.S. et al., 2019; Rendeiro et al., 2013; Lee, Choi, Lee, Shin, & Cho, 2020). For example, supplementation with black rice extract rich in anthocyanins may prevent memory and cognition deficits in mice (Lee et al., 2020). In one study, pure anthocyanins significantly improved spatial working memory, to an extent similar to anthocyanin-rich blueberry powder (Rendeiro et al., 2013). Moreover, anthocyanin extract was observed to improve learning and memory in mice after surgery and significantly reduced neuroinflammation and microglia activation (Zhang et al., 2020). In addition to memory and cognition benefits, sufficient evidence has shown neuroprotective effects of anthocyanins against neurodegenerative disorders, including Alzheimer's (AD) and PD (Winter & Bickford, 2019; Zhang et al., 2019; Li et al., 2020).

Notwithstanding the effect on brain function, underlying mechanisms of action of dietary anthocyanins are not fully understood. Some of the proposed mechanisms include their passage across the blood–brain barrier and modulation of cell signaling pathways and expression of genes involved in neuroinflammation, synaptic plasticity, and neurogenesis, especially in the hippocampus, an area of the brain critical for learning and memory (Hein et al., 2019; Rees, Dodd, & Spencer, 2018). The capacity of anthocyanins to modify cell signaling pathways and expression of a large number of different genes involved in the regulation of various processes like inflammatory responses, redox balance, cell migration, and metabolism has been previously shown, particularly in the vasculature (Krga et al., 2018; Mauray et al., 2012; Rodriguez-Mateos et al., 2019). Several studies have also suggested that anthocyanins could modulate the expression of microRNAs that are post-transcriptional regulators of gene expression (Krga & Milenkovic, 2019; Krga et al., 2018; Milenkovic et al., 2012; Rodriguez-Mateos et al., 2019). Despite the proposed pleiotropic nature of anthocyanins, studies employing holistic approaches that allow examination of the effect on several thousands of targets in the brain are still limited. However, this research would be of relevance to generate a first global picture of the complex mechanisms that could underlie the effects of these bioactives on cognitive function and neurodegenerative diseases.

Apolipoprotein E deficient (ApoE $^{-/-}$) mice have been widely used to study vascular biology and the effects of nutrition, including polyphenols, on the prevention of vascular dysfunction and associated diseases. These mice are characterized by poor lipoprotein clearance, accumulation of cholesterol-rich particles in the blood, increased systemic inflammation, and impaired endothelial function, all promoting the formation of atherosclerotic plaques (Lo Sasso et al., 2016). Numerous studies have shown that dietary supplementation with various polyphenols, including anthocyanins, is highly efficient in decreasing inflammation, improving endothelial function, and reducing the formation of atherosclerotic plaques in the aorta of these mice (Krga & Milenkovic, 2019; Mauray et al., 2012, 2009). In addition to causing disturbances in the periphery, ApoE deficiency also affects proper brain function. Brain-derived ApoE-containing lipoproteins are necessary for synapse development, and their depletion leads to synaptic loss and dysfunction (Lane-Donovan et al., 2016). Moreover, hypercholesterolemia and vascular dysfunction lead to reduced cerebral blood flow and dysregulated cerebrovasculature (Ayata et al., 2013; Lane-Donovan et al., 2016). ApoE$^{-/-}$ mice also exhibit increased blood–brain barrier permeability that allows the entry of different neurotoxic substances, including inflammatory cytokines, and promotes neuroinflammation (Bell et al., 2012; Lane-Donovan et al., 2016). All these effects have been associated with synaptic loss, reduced neurogenesis, and cognitive deficits (Ayata et al., 2013; Lane-Donovan et al., 2016; Zerbi et al., 2014); thus, ApoE$^{-/-}$ mice can also be particularly suitable for examining the impact of polyphenols on brain function.

Taken together, this study aimed to investigate the effects of anthocyanin-rich bilberry extract (BE) supplementation on the global gene expression in the hippocampus of ApoE$^{-/-}$ mice to help the understanding of molecular mechanisms related to the reported neuroprotective effects of anthocyanin-rich food consumption.

2. Materials and methods

2.1. Bilberry extract

Bilberry (*Vaccinium myrtillus* L.) anthocyanin-rich extract, Antho 50®, was provided by Ferlux S.A. (Cournon d'Auvergne, France). It contained 52% (w/w) of anthocyanins expressed as cyanidin-3-glucoside equivalent as previously determined (Mauray et al., 2009). Anthocyanins identified in the extract by HPLC analysis included: delphinidin-3-galactoside (11.69%), delphinidin-3-glucoside (13.98%), cyanidin-3-galactoside (8.85%), delphinidin-3-arabinoside (9.68%), cyanidin-3-glucoside (11.64%), petunidin-3-galactoside (4.18%), cyanidin-3-arabinoside (6.47%), petunidin-3-glucoside (9.10%), peonidin-3-galactoside (0.75%), petunidin-3-arabinoside (2.62%), peonidin-3-glucoside + malvidin-3-galactoside (9.22%), peonidin-3-arabinoside + malvidin-3-glucoside (10.23%) and malvidin-3-arabinoside (1.60%) (**Supplemental figure S1**). The total polyphenol content was 62 g gallic acid equivalent/100 g of BE, as determined by the Folin-Ciocalteu assay (Mauray et al., 2009).

2.2. Animals and diets

Pairs of homozygous ApoE$^{-/-}$ mice were obtained from Jackson Laboratories (Charles River Laboratories, L'Arbresle, France) and interbred to yield the males used in this study. Mice were individually housed in wire-bottomed cages at controlled room temperature (22 ± 0.8 °C) and relative humidity (55 ± 10%), under a 12 h light/dark cycle. The animals had free access to food (control or supplemented with the extract) and water. Animals were handled according to the recommendations of the Institutional Ethics Committee of INRA in agreement with decree N° 87–848. Before the beginning of the experiment, animals were fed a standard breeding diet A03 (Safe, Epinay-sur-Orge, France). At the age of 8 weeks, mice were randomly divided into two groups of 6 animals and fed for 12 weeks, either a control diet or a control diet supplemented with 0.02% anthocyanin-rich BE. A detailed composition of diets is given in Table S1. During the preparation of the diet mixture, the extract was added, and the pellets were prepared. The pellets were prepared every 2 weeks to prevent a decrease in anthocyanins with time (pellets were kept in vacuum bags, in the dark at 4 °C). At the end of the experimental period, mice were anesthetized with sodium pentobarbital at 40 mg/kg of body weight. Hippocampi were collected, immediately frozen in liquid nitrogen, and stored at −80 °C until analyzed.

2.3. Microarray analysis

***RNA extraction and fluorescent labeling*:** Hippocampi stored at −80 °C were homogenized in lysis buffer for total RNA extraction using the SV Total RNA Isolation System (Promega, Madison, WI, USA) as recommended by the manufacturer. Total RNA was extracted from eight hippocampi: four from mice on a control diet and four from mice on a BE-supplemented diet. The quality and integrity of total RNA were checked by 1% agarose gel electrophoresis. The cDNAs were reverse-transcribed from 5 μg of total RNA with 1 μL of oligo(dT) and 1 μL of random primer and labeled with CyTM3- or CyTM5-dCTP using the ChipShot^{TM} Direct Labeling System (Promega). The synthesized, labeled cDNA was subsequently purified using the ChipShot^{TM} Membrane Clean-Up System (Promega). The labeling efficiency and the quantity of cDNA were assessed by measuring the absorbance at 260, 550, and 650 nm with the ND-1000 spectrophotometer (Nanodrop, Wilmington, DE, USA).

Hybridization: Samples of cDNA were hybridized to mouse Operon

microarrays (OpArrays™, Operon Technologies, Alameda, CA, USA). Mouse Genome Array-Ready Oligo Set version 4.0 contains 35,852 longmer probes representing around 25,000 genes and 38,000 gene transcripts. Eight microarrays were used for a total of four independent comparisons. Hybridization was performed for eight hours at 42 °C in the Ventana hybridization system (Ventana Medical Systems, S.A, Illkirch, France). Slides were washed twice in 2X saline sodium citrate and 0.1X saline sodium citrate at room temperature and scanned with the Agilent G2505 microarray scanner system (Agilent Technologies, Inc., Santa Clara, CA, USA) at 5 μm resolution.

Image and data analysis: These steps were performed as previously detailed (Auclair et al., 2009). Briefly, the ImaGene 6.0 software (BioDiscovery, Inc, Proteigene, Saint-Marcel, France) was used to acquire the signal and background intensity values for each spot in both channels. Data were log base-2 transformed and corrected for systemic dye bias with the Lowess normalization using GeneSight 4.1 software (BioDiscovery, Inc, Proteigene). The log-ratio between BE-supplemented and control samples was analyzed with Student's t-tests using the free R 2.1 software (http://www.r-project.org), and Bonferroni correction was performed to remove false positives. Genes identified by these criteria were considered as differentially expressed between two nutritional conditions.

2.4. Bioinformatics analyses

Nutrigenomic data were analyzed with different bioinformatics tools. Gene expression profiles in two studied nutritional groups were compared by partial least squares-discriminant analysis (PLS-DA) using the MetaboAnalyst web-based software (https://www.metaboanalyst.ca/) (Chong, Wishart, & Xia, 2019). Ensembl database (http://www.ensembl.org/index.html) was used for the identification of official symbols and names of differentially expressed genes. To identify biological processes associated with the identified differentially expressed genes, Gene Ontology (GO) analysis was performed using the DAVID database, version 6.8 (https://david.ncifcrf.gov/) (Huang, Sherman, & Lempicki, 2009), and the obtained results visualized as a TreeMap chart by REVIGO platform (http://revigo.irb.hr/) (Supek, Bošnjak, Škunca, & Šmuc, 2011). Process network analysis was performed by employing the text mining algorithm of MetaCore software suite (Clarivate Analytics, Philadelphia, PA, USA, https://portal.genego.com). Overrepresented pathways were identified using GeneTrail2, version 1.6 (https://genetrail2.bioinf.uni-sb.de/workflow.html) (Stöckel et al., 2016) that allowed the analysis of transcriptomic data by accessing the BioCarta and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases. Enriched pathways were also identified using the MetaCore™ software suite (Clarivate Analytics, Philadelphia, PA, USA; https://portal.genego.com) and results were combined with GeneTail2-obtained data. Interactions between functional groups of genes were analyzed with Cytoscape software (version 3.7.2; https://cytoscape.org/), using the ClueGO (Bindea et al., 2009). Comparative Toxicogenomics Database (http://ctdbase.org/) that provides information on chemical-gene/protein interactions, gene-disease, and chemical-disease relationships was used to further examine our differentially expressed genes and identify genes in common with disease-related ones and those inferred with common drugs used to treat cognition disorders, AD and PD.

Analysis of the protein–protein interactions between the proteins encoded by differentially expressed genes, including their neighboring proteins, was performed by STRING database, version 11.0 (https://string-db.org/) (Szklarczyk et al., 2019), under the following settings: network edges – confidence; active interaction sources – text-mining, experiments, databases, and co-expression; minimum required interaction score – high confidence (0.700); the maximal number of displayed interactions – 1st shell: query proteins only and 2nd shell: no more than 5 interactions. The obtained protein network was organized in three clusters of functionally connected proteins by kmeans clustering, and the overrepresented pathways within the clusters were identified by KEGG. OmicsNet (https://www.omicsnet.ca/) (Zhou & Xia, 2019) was used to identify transcription factors and miRNA-networks potentially involved in regulating the observed nutrigenomic effect. Potential binding between top transcription factors identified using OmicsNet and some of the most abundant anthocyanins in the studied BE, was examined by molecular docking. The 3D structures of proteins were retrieved from the Protein Data Bank (www.rcsb.org) and structures of anthocyanins from the PubChem database (https://pubchem.ncbi.nlm.nih.gov). Docking calculations were performed using the DockingServer (https://www.dockingserver.com/web) and 1click docking from Mcule.org (https://mcule.com/apps/1-click-docking/).

2.5. Statistical analysis

Prism software, version 8.1.1 (GraphPad, La Jolla, CA, USA) was used for statistical analysis. Body weight and food intake data were analyzed by *t*-test to compare the effect of BE-supplemented with the control diet. The $p \leq 0.05$ was considered significant.

3. Results

3.1. The effect of BE supplementation on body weight and food intake

There were no significant differences in body weight between ApoE$^{-/-}$ mice fed the control or BE-supplemented diet during the 12 weeks. Food intakes did not differ between the two studied groups in the last weeks of experimental period (Fig. 1).

3.2. Nutrigenomic effect of anthocyanin-rich BE extract

A comparison of gene expression profiles in the hippocampus of ApoE$^{-/-}$ mice fed the control or BE-supplemented diet by PLS-DA showed a distinct separation between the two nutritional groups (Fig. 2). Gene expression analysis demonstrated that anthocyanin-rich BE supplementation induced significant changes in hippocampal gene expression. There were 2466 probes identified as differentially expressed between the two nutritional groups that corresponded to 1698 different genes. Of these, 611 genes were identified as down-regulated by BE supplementation and 1087 as upregulated. The fold-change values varied from −1.98 to 1.95 (Table S2) with an average fold-change of −1.16 for the downregulated genes and 1.22 for the upregulated genes.

3.3. Bioinformatic analyses of the nutrigenomic effect of anthocyanin-rich BE extract

The nutrigenomic data were further analyzed with different bioinformatics tools to define the biological functions of the identified differentially expressed genes. A global GO analysis was firstly conducted to determine biological processes associated with these genes. This analysis revealed that anthocyanin-rich BE supplementation modulated the expression of genes involved in regulating various biological processes such as brain development, protein phosphorylation, cell to cell adhesion, response to hypoxia, and apoptosis (Supplemental Fig. 2). Gene network analysis, obtained by the text mining approach, was further performed for a more in-depth examination and interpretation of biological functions associated with differentially expressed genes. These results suggested that anthocyanin-rich BE altered the expression of genes regulating development, cell signaling, inflammation, cytoskeleton organization, cell cycle, and muscle contraction (Fig. 3). More specifically, the development network cluster included pathways associated with neurogenesis like axonal guidance, regulation of telomerase length, and Hedgehog signaling. Within the cell signaling network were pathways like CREM signaling, androgen receptor, progesterone, and estrogen receptor-1 signaling pathways. The inflammation network cluster contained genes involved in the regulation of

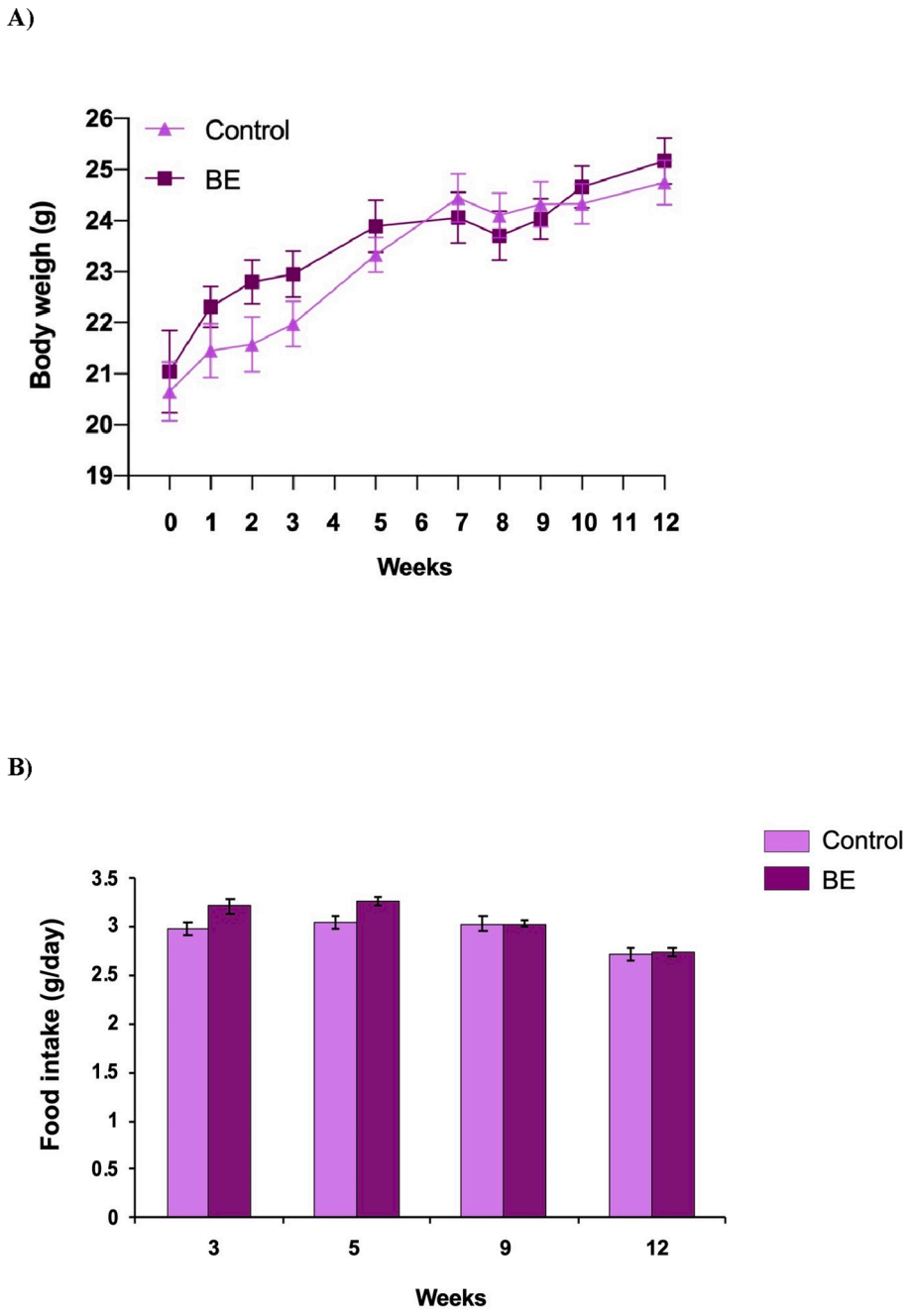

Fig. 1. Body weight changes (A) and food intake (B) in ApoE-/- mice with or without dietary supplementation with anthocyanin-rich BE for 12 weeks.

leukocyte chemotaxis and macrophage migration inhibitory factor (MIF) signaling, while genes of the cell cycle network cluster were linked with the regulation of S phase, G2-M phase, and mitosis. Further analysis of transcriptomic data by KEGG, BioCarta databases, and MetaCore, revealed cellular pathways associated with the genes which expression was significantly modulated by BE supplementation. The over-represented pathways were related to cell signaling, inflammation and cell motility, neuro-related processes, metabolism, cell growth and death (Fig. 4A). The most significant cell signaling pathways included PI3K-Akt signaling pathway represented with 33 genes (e.g., *ITGB7*, *IGF1*, *GH*, *PIK3R5*, *PHLPP2*, *CREB3L1*), Jak-STAT signaling pathway with 12 (e.g., *IL4*, *IL13RA1*, *IFNGR2*, *IL2*, *IL23R*), HIF-1 signaling pathway with 11 (e.g., *IGF1R*, *IFNGR2*, *FLT1*, *NOX1*, *EIF4E*) and TNF signaling pathway with 9 genes (e.g., *TAB2*, *CCL20*, *VCAM1*, *NOD2, TRAF3*). Among neuro-related pathways, the highest number of genes modulated by anthocyanin-rich bilberry extract were identified in Parkinson's disease (e.g., *PARK7*, *NDUFB9*, *NDUFV2*, *DRD1*, *DRD2*, *GNAI2*), constitutive and regulated NMDA receptor trafficking (e.g., *GRIN2A*, *GRIN2D*, *DRD1*, *PPP1CA*, *PRKAR2A*, *ADCY1*), regulation of AKT(PKB)/GSK3 beta cascade in bipolar disorder (e.g., *NTRK1*, *TNTRK2*, *NRG1*, *RET*, *IGF1R*), nicotine signaling in dopaminergic neurons (e.g., *ADRB1*, *ADRB3*, *GNAI2*, *ADCY1*) and CREB signaling pathways (e.g., *PDYN*, *CAMK4*, *PPP1CA*, *PRKAR2A*, *ADCY1)*. Overrepresented pathways related to inflammation and cell motility included cytokine-cytokine receptor interaction (represented with genes like *IL4*, *IL2*, *CCL20*, *CXCL13, IL13RA1*, *IFNGR2*, *TNFRSF9*, *CX3CR1, FLT1*), sublytic effects of membrane attack complex (e.g., *TRAF6, MCU*, *EIF2AK3*, *PLA2G4A*, *HSP90B1, HSP27*), platelet-activating factor/PTAFR pathway signaling (e.g., *LSP1*, *NFKB2*, *PLCB2*, *NFATC1*), Th1 and Th2 cell differentiation (*PLAT*, *DRD1*, *PPP1CA*, *PRKAR2A*, *PLCB4*) as well as cytoskeleton

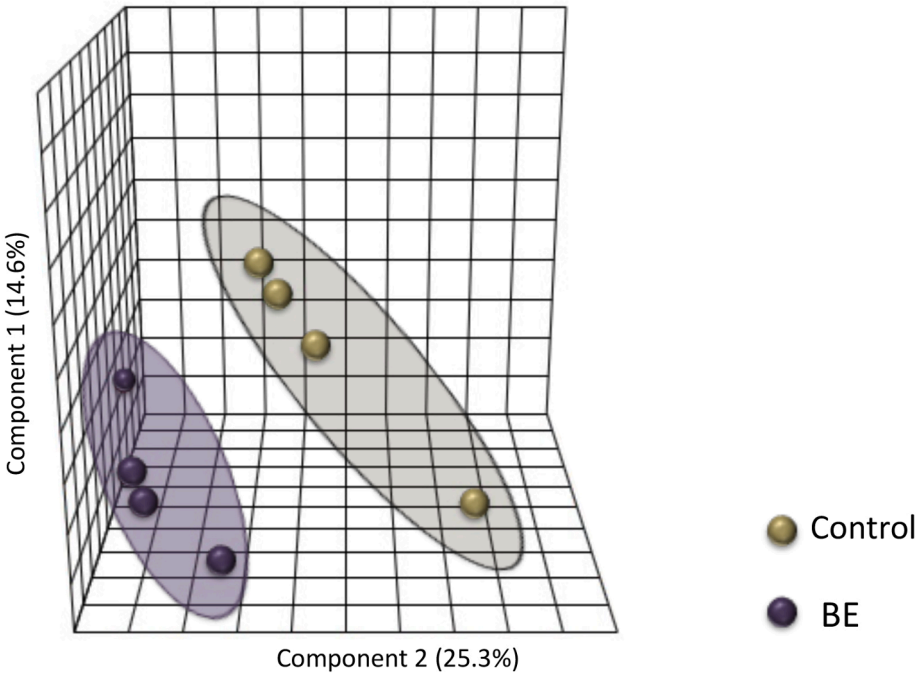

Fig. 2. PLS-DA separation of gene expression profiles in the hippocampus of ApoE-/- mice fed control or anthocyanin-rich BE-supplemented diet.

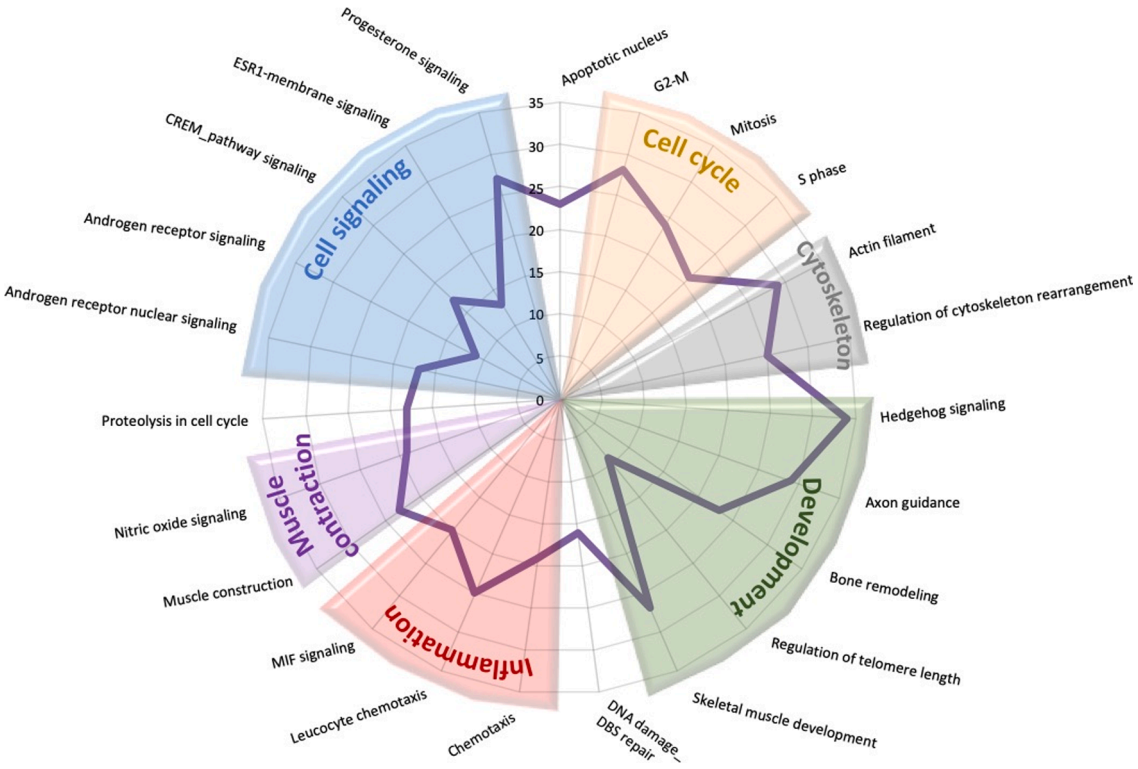

Fig. 3. The most significant process networks of differentially expressed in the hippocampus of ApoE-/- mice in response to anthocyanin-rich BE supplementation.

remodeling-role of PKA in cytoskeleton reorganization (e.g., *MLCK*, *LASP1, PP1CA*, *ADCY1*). Additionally, numerous genes were associated with the regulation of metabolism as in purine metabolism (e.g., *ADA, NME2*, *PNP*, *NUDT2*, *POLR3G*), pyrimidine metabolism (e.g., *AK3*, *PNP*, *NUDT2*, *POLR2C*), and oxidative phosphorylation pathways (e.g., *NDUFA11*, *NDUFS3*, *ATP5C1, ATP5J2*, *COX10*). As presented in Fig. 4B, these pathways are interconnected among them.

3.4. Genes associated with neurodegeneration and treatment drugs

The list of differentially expressed genes was analyzed in the Comparative Toxicogenomics Database to identify genes concomitant with different neurological disorders, including cognitive dysfunction, cognitive disorders, AD and PD. This analysis showed that a high number of genes identified as modulated by anthocyanin-rich extract are associated with these disorders. Over 1000 genes have overlap with

A)

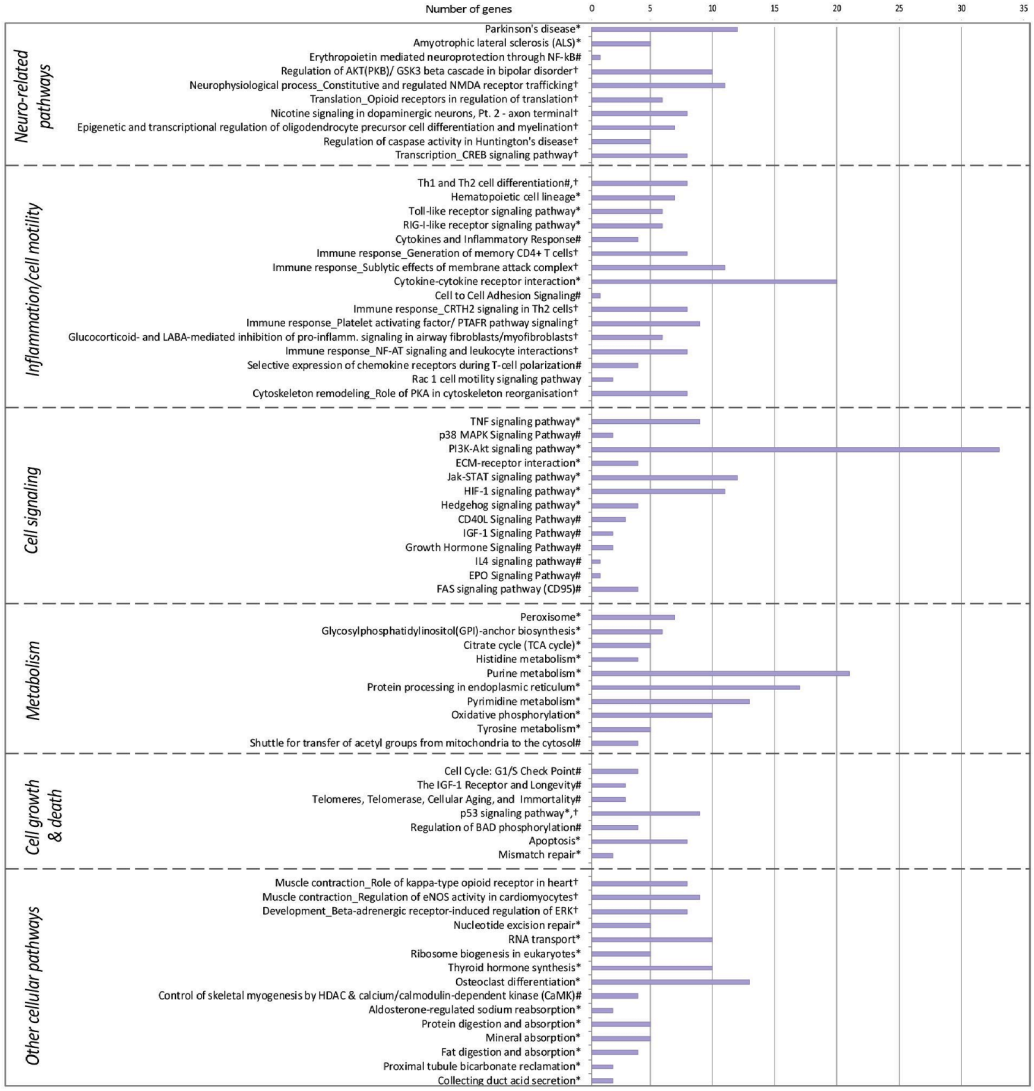

Fig. 4. Pathways enriched with differentially expressed genes in the hippocampus of ApoE-/- mice in response to anthocyanin-rich BE supplementation. Results obtained using BioCarta (#), KEGG (*) database and MetaCore (†) presented as a histogram **(A)** and network **(B)**.

inferred genes associated with both AD and PD (Fig. 5A). Among the common genes are *IL4, CD33, TREM2, INPP5, BIN1, ADRB1* and *MYH8*. Regarding cognitive function, over 900 genes are similar to inferred genes associated with cognitive disorders and cognitive dysfunction, like *ACADL, ACTR6, BMP2, CDC40, GDF11, IL4,* and *NOL10* (Fig. 5B). This analysis suggests that genes identified as modulated by anthocyanin-rich extract could be involved in the occurrence and development of these neurological-related dysfunctions and diseases. Furthermore, we analyzed the intersecting genes between our differentially expressed genes and genes inferred with common drugs for treating AD, PD, and cognitive dysfunctions, including donepezil, galantamine, rivastigmine, memantine, levodopa, carbidopa, safinamide, ropinirole, pramipexole, benztropine, trihexyphenidyl, rasagiline, and selegiline. Among these drugs, we observed an overlap of genes between our differentially expressed genes and levodopa (*AGT, ATXN2, CYP1A1, DRD1, DRD2, GABRG2, GAP43, NSF, PARK7, PDYN, SLC11A2,* and *STMN1*), while little overlap was observed for donepezil, ropinirole, and pramipexole.

3.5. Protein-protein interaction prediction

Differentially expressed genes were further analyzed to create a network of protein–protein interactions and identify central nodes presenting the highest number of functional connections, whose modulation may significantly impact protein–protein interactome and cellular function. The obtained network included 1702 nodes (proteins) and 2992 edges (predicted functional associations). Within the network (Fig. 6A), proteins were organized in three distinctive clusters that corresponded to proteins mainly involved in ubiquitin-mediated proteolysis (green), PI3K-Akt signaling (red), and neuroactive ligand-receptor interactions (blue). Proteins that showed the highest degree of

B)

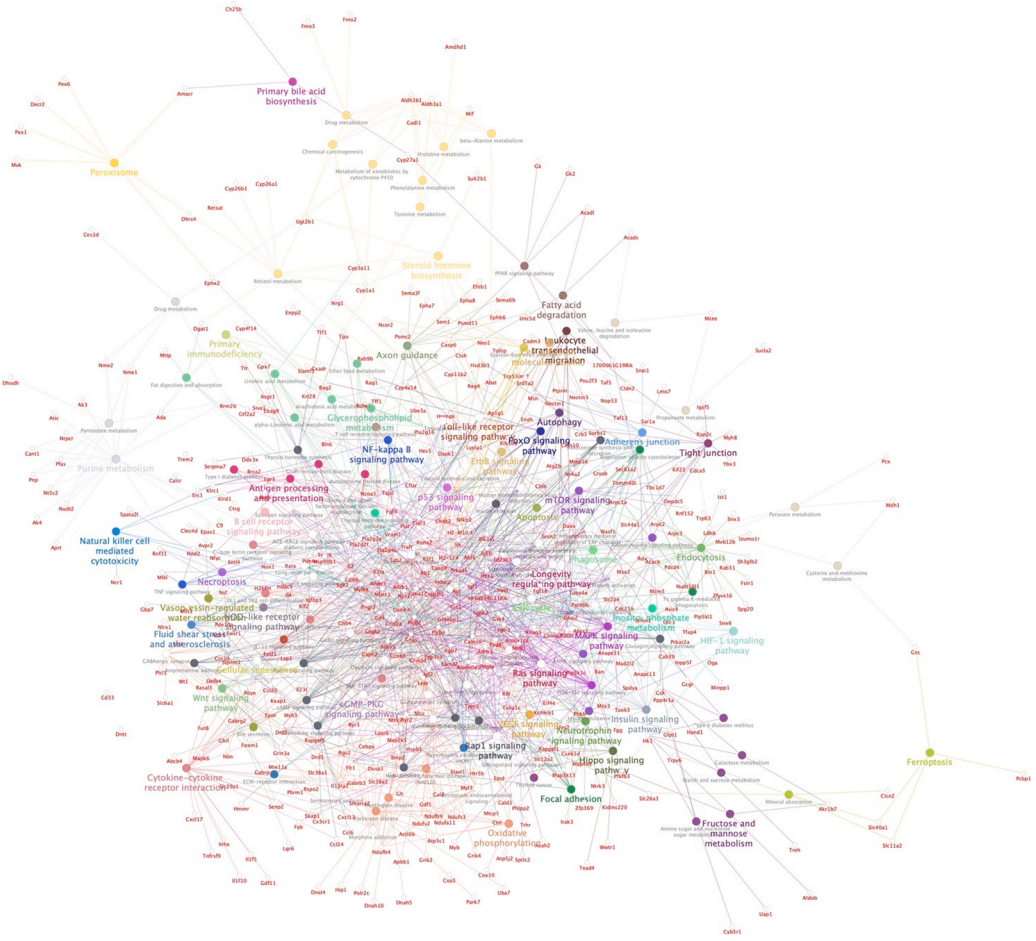

Fig. 4. (*continued*).

interconnectivity included CUL2, KEAP1, POLR2C, and GNB4 (Fig. 6B).

3.6. Identification of transcription factors and microRNAs involved in the observed nutrigenomic modification

Finally, to identify potential transcriptional and post-transcriptional (microRNAs) regulators that could mediate the observed nutrigenomic effect of anthocyanin-rich BE supplementation, the differently expressed genes were analyzed by the OmicNet platform. Among the most significant putative transcriptional regulators were SP1, NFKB1, TRP53, JUN, and RELA, while the most significant potentially involved miRNAs included mir-124-3p, mir-340-5p, mir-181a-5p, mir-329-3p, mir-15a-5p, and mir-9-5p (Fig. 7). Multi-layered 3D examination of regulatory networks showed a high level of interconnectivity between regulatory elements (either transcription factor or miRNA) and differentially expressed genes (Fig. 7).

3.7. Interaction between major anthocyanins and transcription factors

Following identification of the potential transcription factors, we searched for possible interactions between BE anthocyanin, delphinidin-3-galactoside present in the circulation, and proteins of interest: JUN, RELA, SP1, p53 (Fig. 8). This anthocyanin presented potential strong interactions with proteins of interest, with the binding affinity varying between −7.2 and −8.52 kcal/mol. We observed binding energy of −7.2 kcal/mol for interaction between delphinidin-3-galactoside and JUN, −8.16 kcal/mol for interaction with RELA, −8.52 for SP1, and binding energy of −7.25 kcal/mol for p53. Moreover, in-silico docking analysis also showed that cyanidin-3-glucoside can interact with CREBP transcription factor (-8.1 kcal/mol) or RELA (-7.1 kcal/mol) (Supplemental Fig. 3). Therefore, these results suggest that anthocyanins present in the extract can interact with proteins involved in gene expression regulation, presenting a possible mode of action of anthocyanins.

4. Discussion

In this study we showed that 12-week dietary supplementation with anthocyanin-rich BE changed the global gene expression profile in the hippocampus of ApoE$^{-/-}$ mice. Bioinformatic analyses suggested that the identified 1698 differentially expressed genes could be involved in various cellular and molecular processes, including inflammation, neuronal function, metabolic processes and signal transduction, that are involved in cognitive function and neurodegenerative disorders like AD and PD. Furthermore, some transcription factors and miRNAs are suggested as potential mediators in the observed nutrigenomic effect.

The dose used in this study can be nutritionally achievable in humans and can exert vasculo-protective effects. We have previously shown that the same extract exerts an antiatherogenic effect in ApoE-/- mice after dietary supplementation at 0.02% in the diet (Mauray et al., 2009) and can also modulate the expression of genes associated with atherosclerotic effects in the aorta, as observed using microarray analysis (Mauray et al., 2012). The supplementation of the diet with 0.02% of this extract (i.e., around 0.01% of anthocyanin glycosides) may correspond to an equivalent human intake of about 30 mg of anthocyanidins per day

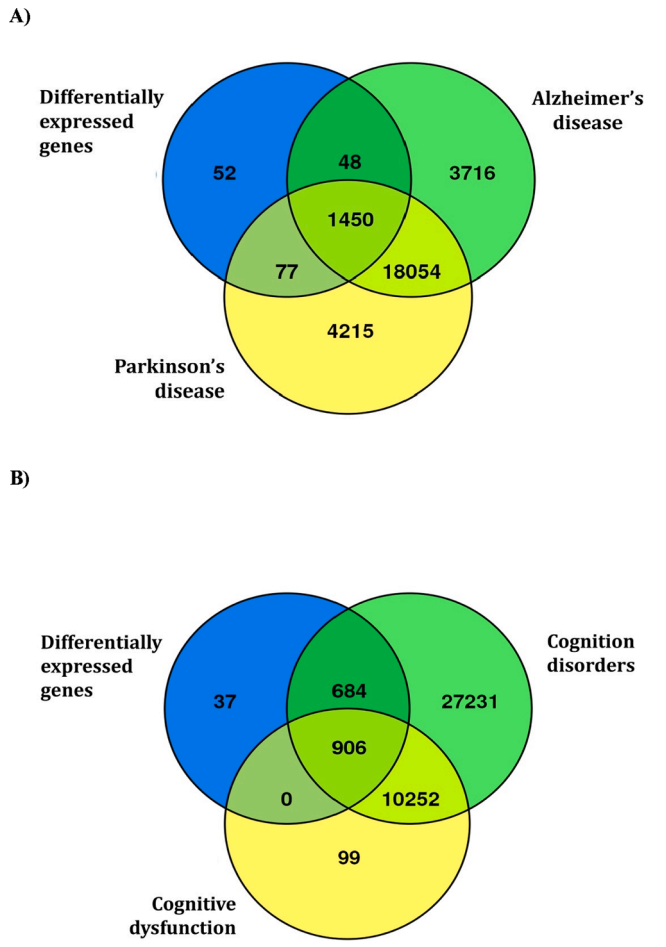

Fig. 5. Venn diagrams showing the overlap between the identified differentially expressed genes and genes implicated in Alzheimer's and Parkinson's diseases **(A)** or cognition disorders and cognitive dysfunction **(B).**

when expressed on the basis of diet content (for a human food intake estimate of 500 g of dry weight). We have previously reported that the estimated mean intakes of anthocyanins in Europe range from 19.8 to 64.9 mg/day for men and from 18.4 to 44.1 mg/day for women, while in the United States, the reported mean consumption is 12.6 mg/day for women and 10.5 mg/day for men (Krga & Milenkovic, 2019). Therefore, the dose used in this study is nutritionally relevant and has been shown to exert vasculo-protective effects and genomic modification in the aorta.

The notion of neuroinflammation includes the response of immune brain cells toward infections but also infiltration of the brain by cells of the innate and adaptive immune systems. An inflammatory response, particularly chronic neuroinflammation, is considered detrimental and damaging to nervous tissue in the central nervous system (CNS) by increased levels of cytokines, proteases, glutamate, free radicals, and activation of glial cells (de Araújo Boleti et al., 2020). This inflammatory process within the brain is one of the key factors triggering different neurological diseases, such as AD, PD, stroke, and cognitive dysfunction (de Araújo Boleti et al., 2020; Zhao et al., 2019). Under inflammatory conditions, activated microglial cells can increase levels of proinflammatory cytokines (e.g., tumor necrosis factor-α, interleukin-1β) and nitric oxide, which are considered hallmarks of neurodegenerative diseases, and in turn induce neuronal death. Neurodegenerative diseases are chronic, progressive, and incapacitating disorders that affect patients' quality of life and frequently result in death. Most of them have no therapy, and the existing treatments aim to ease the symptoms and delay their progression (de Araújo Boleti et al., 2020). In our study, we observed that anthocyanin-rich BE modulated a certain number of genes involved in the regulation of inflammation, which expression profile is suggestive of an anti-inflammatory effect. This observation suggests that these bioactives could lower inflammation and consequently prevent or delay the onset of neurodegenerative diseases and cognitive disorders. Moreover, interrogation of genes associated with AD, PD and cognitive dysfunction and comparison with our differentially expressed genes have revealed high similarity among them. Our hypothesis is in agreement with few studies that revealed the capacity of anthocyanin-rich foods to improve cognitive function and delay AD and PD onset. In a recent randomized, double-blind placebo-controlled parallel study with middle-aged, overweight adults, anthocyanin-rich *Aronia melanocarpa* extract consumption for 24 weeks showed beneficial effects on cognitive performance and blood pressure in individuals at risk of cognitive decline (Ahles et al., 2020). In a mice model of AD, it has been observed that supplementation of a diet with an anthocyanin-rich extract from *Hibiscus sabdariffa* could prevent AD by exerting anti-inflammatory, anti-acetylcholinesterase, antioxidant, and anti-amyloidogenic activities (El-Shiekh, Ashour, Abd El-Haleim, Ahmed, & Abdel-Sattar, 2020). In addition, there are also data, though limited, suggesting that anthocyanins and other polyphenols could similarly contribute to prevent or delay PD (Haider, Madiha, & Batool, 2020; Jung & Kim, 2018).

In this study we showed that supplementing mice diet with a nutritionally relevant dose of anthocyanin-rich extract could significantly change the global gene expression profile. Therefore, this observation supports the capacity of anthocyanins and other bioactives present in the extract to exert *in vivo* a significant genomic effect in the brain. Interestingly, this finding nicely completes and extends the very few studies previously reporting that these bioactives can modulate gene expression *in vivo* in the brain. For example, bilberry anthocyanins were reported to modulate the expression of inflammatory factors (TNF-α, NF-κβ, IL-1β, IL-6, iNOS, COX-2, and CD33), chemokine receptor CX3CR1, microglia homeostatic factors (TREM2 and TYROBP) and Toll-like receptors (TLR2 and TLR4) (Li et al., 2020). Supplementation with purple sweet potato naturally occurring anthocyanins can promote hippocampal brain-derived neurotrophic factor expression by activation of AMP-activated protein kinase (Zhuang et al., 2019) and decrease the expression of TNF-α, IL-1β, suppressor of cytokine signaling 3, allograft inflammatory factor 1 and galectin-3 (Qin et al., 2019). Of note, these rare studies used the targeted approach, which encompasses the analysis of the expression of a few specific genes. It has been shown that anthocyanin *in vivo* can modulate a large number of genes, over a few hundred (Mauray et al., 2010, 2012; Rodriguez-Mateos et al., 2019). Therefore, the use of an untargeted approach, as performed in this study, can enable identifying all the genes whose expression can be modulated by anthocyanins and appears as the most adapted method to determine, as precisely as possible, the molecular mechanisms underlying their health properties.

Bioinformatic analysis of identified differentially expressed genes suggests that anthocyanin-rich BE could modulate the expression of genes involved in regulating different cellular functions. A group of gene networks and cellular pathways involved in the control of inflammation has been revealed. Among the genes and pathways identified is a group of genes involved in chemotaxis. For example, we observed a decrease in the expression of a gene coding for chemokine (C-C motif) ligand 20 (CCL20). Increased brain *CCL20* was detected in response to neuron-derived extracellular α-synuclein stress and contributed to neuroinflammation in neurodegenerative diseases (Lee, Kim, & Lee, 2010). Accordingly, targeting CCL20 using antibodies was shown to reduce inflammation in mice (Liao, Zhang, Gu, & Sun, 2020). We also observed a decrease in the expression of genes encoding CXC chemokine ligand 13 (CXCL13) and CX3C chemokine receptor 1 (CX3CR1). The CXCL13 has been implicated in the recruitment of B cells to the CNS in different neuroinflammatory diseases, but also neurodegeneration and cognitive impairment (Shen et al., 2020). Reduced expression of chemokine receptor CX3CR1 by small interfering RNA was shown to decrease levels of

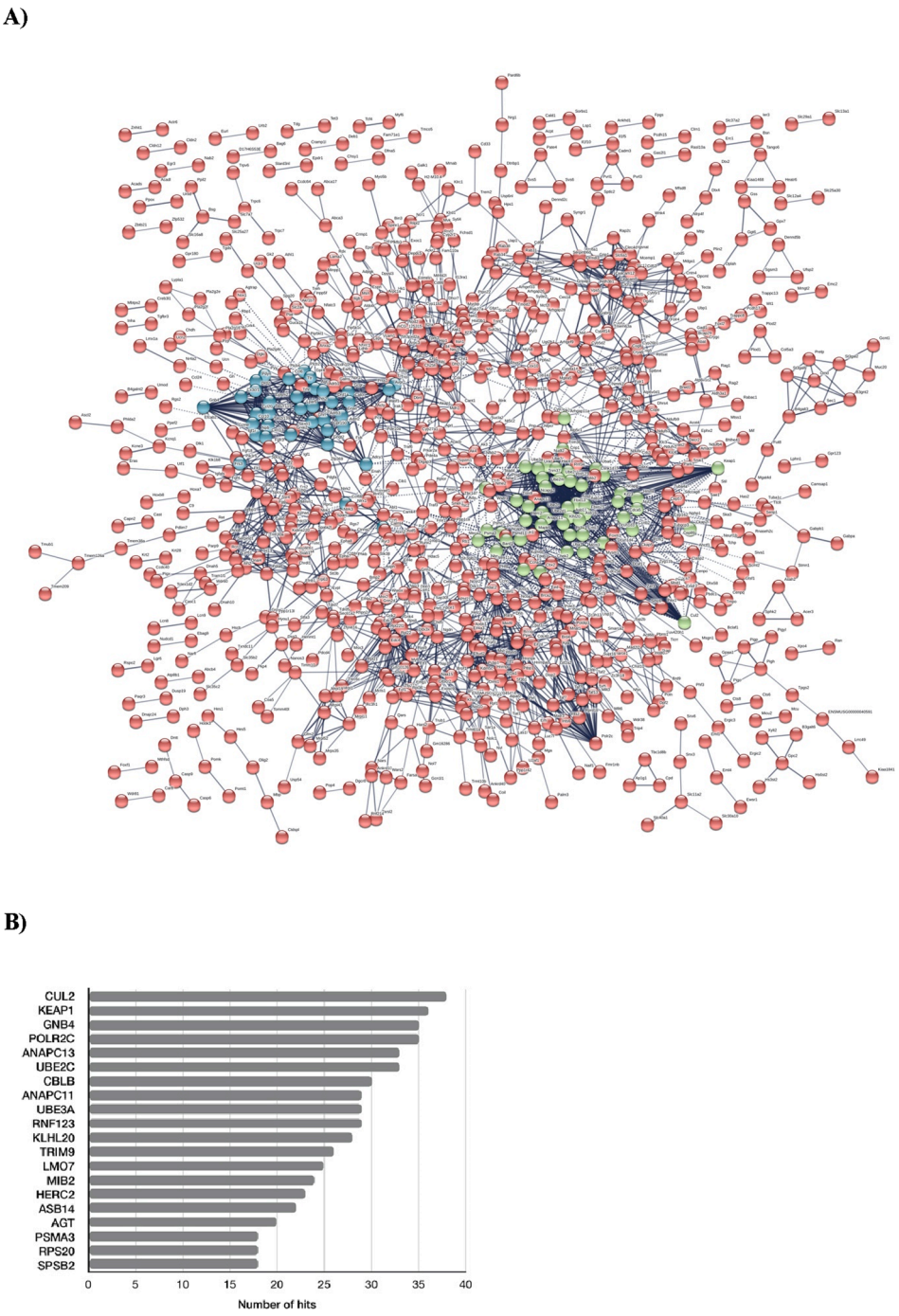

Fig. 6. Interactions between proteins encoded by genes modulated by anthocyanin-rich BE-supplementation **(A)**, with top proteins forming the highest number of interactions **(B)**. Proteins are grouped in clusters, green denotes protein mainly involved in ubiquitin-mediated proteolysis, red in PI3K-Akt signaling and blue in neuroactive ligand-receptor interactions.

proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6 in a mouse brain after ischemia (Liu et al., 2015). Moreover, reduced hippocampal *CX3CR1* levels, downregulation of several proinflammatory cytokines, and reversed cognitive dysfunction were observed in an AD animal model after administration of bilberry anthocyanins (Li et al., 2020). Here, we also detected an increased expression of *IL4* that codes for an anti-inflammatory cytokine produced by Th2 cells, whose capacity to attenuate age- and AD-associated neuroinflammation was reported in several animal studies (Boccardi et al., 2019; Maher, Nolan, & Lynch, 2005). Therefore, the modulation of expression of these and other chemokines and their receptors in the hippocampus following supplementation with anthocyanin-rich BE suggests its anti-neuroinflammatory action and protective effect against neurodegenerative disorders.

Our bioinformatic analysis also suggests that BE could modulate the expression of several genes involved in different metabolism-related pathways such as oxidative phosphorylation (OXPHOS), purine, and pyrimidine metabolism. The OXPHOS is a metabolic pathway responsible for the majority of ATP production in the brain that powers cell signaling and neuronal activity processes (Bergman & Ben-Shachar, 2016). Altered expression of several components of the OXPHOS system, such as subunits of mitochondrial complex 1 and ATP synthases,

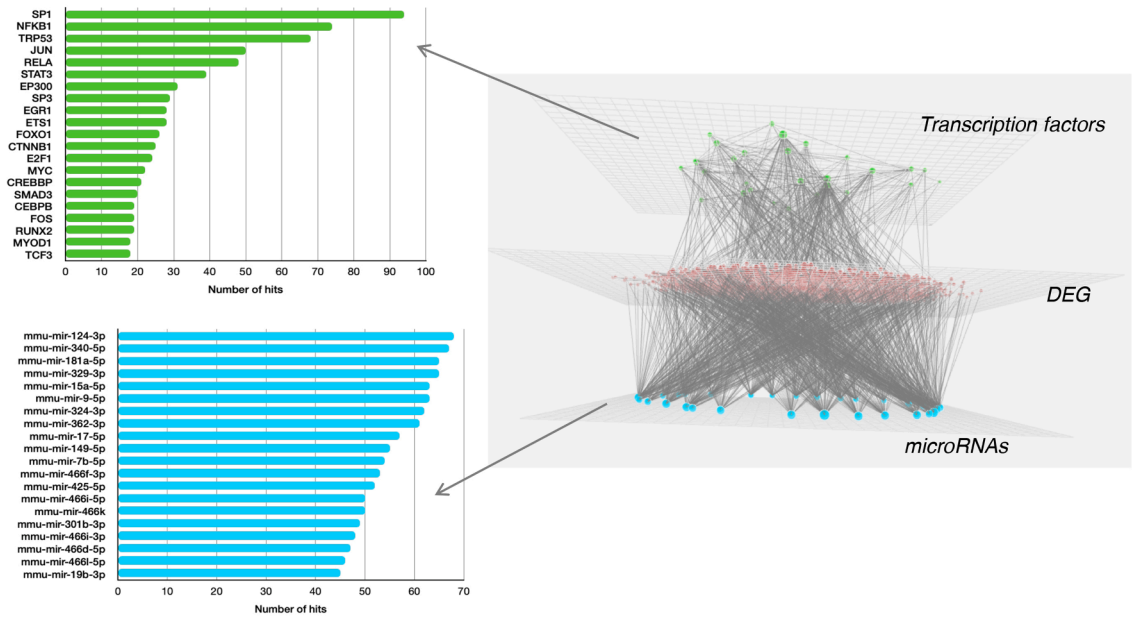

Fig. 7. Top 20 putative transcription factors and miRNA potentially regulating nutrigenomic effect of anthocyanin-rich BE supplementation in the hippocampus of ApoE-/- mice with the multi-layered layout of regulatory networks of transcription factors and miRNAs. Green nodes represent transcription factors, light blue nodes-miRNAs, red nodes-differentially expressed genes.

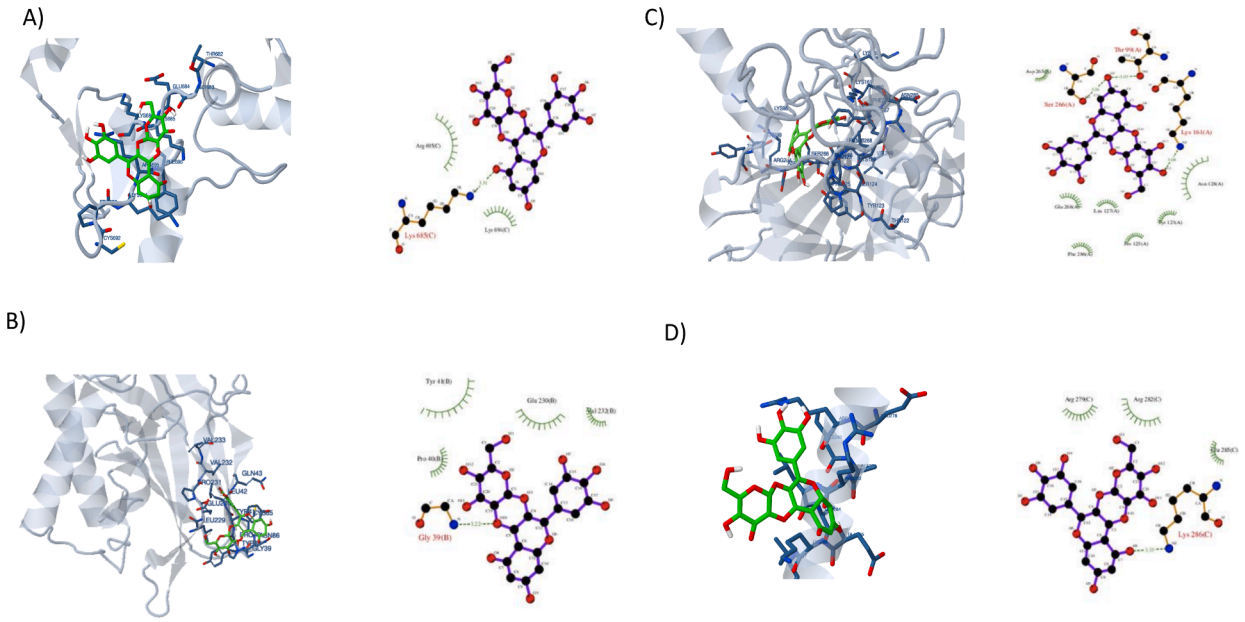

Fig. 8. Docking analysis of major BE anthocyanin, delphinidin-3-galactoside, with the top transcription factors identified in bioinformatics analysis. Each left image shows the strongest binding conformation and each right the interaction analyses between the ligand and A) SP1, B: RELA, C: TRP53, D: JUN.

have been described in the AD brain and may be associated with disturbed energy production and neuronal dysfunctions observed in this condition (Adav, Park, & Sze, 2019; Canchi et al., 2019). Nucleotides also participate in energy metabolism as well as regulation of neuronal activity, blood–brain barrier permeability and cognitive function. Additionally, deregulated expression of several genes involved in their metabolism have been described in the brain of AD and PD subjects (Ansoleaga et al., 2015; Garcia-Esparcia, Hernández-Ortega, Ansoleaga, Carmona, & Ferrer, 2015). Thus, these data suggest that by modulating the expression of genes related to these metabolic pathways, anthocyanin-rich BE consumption might affect cellular metabolism and exert neuroprotective effects.

Among the identified differentially expressed genes were also those implicated in PI3K-Akt signaling that manages various complex events involved in regulating microglia neuroinflammatory responses, metabolism, and neuronal survival (Cianciulli et al., 2020). PI3K-Akt dysregulation has been observed in different neurodegenerative diseases, including AD, PD and Huntington's diseases, and recent evidence suggests the preventive and therapeutic potential of the modulation of this signaling in these pathologies (Cianciulli et al., 2020; Rai et al., 2019). PI3K-Akt was also identified as a notable pathway in our protein–protein interaction analysis. This analysis also revealed several genes encoding different enzymes involved in ubiquitin-mediated proteolysis as potentially important regulatory nodes. The ubiquitin–proteasome system is

essential for the degradation of regulatory and damaged proteins and control of synaptic plasticity and memory, while its dysregulation has been associated with protein accumulation, aberrant synaptic function, and cognitive deficits in different neurodegenerative disorders (Olabarria et al., 2019). Among genes encoding ubiquitination-associated enzymes is *UBE3A* (ubiquitin-protein ligase E3A), found upregulated by BE. UBE3A expression was reported significantly decreased in the hippocampus of cognitively impaired aged rats (Fletcher et al., 2014), and its neuronal loss was associated with synaptic dysfunction in an AD mouse model (Olabarria et al., 2019). Thus, modulation of the expression of this and other genes involved in ubiquitin-mediated proteolysis by anthocyanin-rich BE suggests its potential impact on synaptic function.

In line with this hypothesis, the bioinformatic analysis has also revealed a group of genes involved in cellular pathways of AD and PD development. For example, a group of genes coding for glutamate receptors has been identified. L-Glutamate is the most abundant neurotransmitter in the mammalian CNS that mediates excitatory synaptic transmission (Zhou & Danbolt, 2014). It interacts with both ionotropic and metabotropic receptors, which belong to the group of glutamate receptors involved in various processes, from learning and memory to neurodegeneration such as AD, PD, and Huntington's disease (Serwach & Gruszczynska-Biegala, 2019). Among glutamate receptor coding genes is *GRIN3A,* for which there is growing evidence of its role in various disorders of the CNS, including Huntington's disease and cocaine addiction (Kehoe, Bernardinelli, & Muller, 2013). These receptors are part of glutamatergic synapses, and in the brain, over 40% of neuronal synapses are glutamatergic, with disturbances in glutamatergic function identified as implicated in the pathophysiology of AD (Bukke et al., 2020). We also identified a group of genes involved in the endocannabinoid signaling pathway. The endocannabinoid system plays an essential modulatory role in the endocrine, immune, and brain tissue and includes elementarily G protein-coupled receptors, known as cannabinoid receptors, and the endogenous agonists of these receptors, known as endocannabinoids. The endocannabinoid system constitutes a crucial player in developing various neuropathological states, including depression, AD, PD, multiple sclerosis, and epilepsy (Charytoniuk et al., 2020). Together with other pathways identified as involved in the regulation of brain function, we can suggest that anthocyanin-rich BE modulate the expression of genes involved in cellular pathways regulating neuronal function. The expression profile of these genes is consistent with the capacity of the anthocyanin-rich BE to maintain neuronal function.

In this study, we further performed bioinformatics analysis on the gene expression data to identify putative transcription factors whose activity could be modulated by anthocyanin-rich BE supplementation and associated with the observed changes in the hippocampal gene expression. Among the identified transcription factors was SP1 that regulates the expression of key genes linked with AD pathology and contributes to cognitive dysfunction (Subaiea, Adwan, Ahmed, Stevens, & Zawia, 2013). Additionally, both NFKB1 and RELA subunits of the NF-κB transcription factor were identified. The NF-κB is best known for its role in inflammation but is also implicated in synaptic plasticity, learning, and memory, and its dysregulation has been reported in neurodegenerative diseases (Snow & Albensi, 2016). Anthocyanin-rich extracts were previously reported to improve memory and reduce neuroinflammation by inhibiting NF-κB activation in the brain of high-fat diet-fed (Snow & Albensi, 2016) and LPS-treated mice (Khan M.S. et al., 2019). Among the identified transcription regulators were also JUN and p53, which modulate hippocampal gene expression during long-term potentiation (Mitchnick et al., 2016) and are implicated in neuronal loss and cognitive deficits in different neurological disorders (Szybińska & Leśniakx, 2017; Tang et al., 2020). Recently, anthocyanins from chokeberry were shown to alleviate the age-associated cognitive decline and response capacity in senescence-accelerated mice in part by reducing brain p53 and p-p53 levels (Wu et al., 2019). Thus, these results suggest that dietary supplementation with anthocyanin-rich BE could modulate the activity of various transcription factors and consequently alter the expression of genes associated with maintaining cognitive function, memory, and prevention of neurodegenerative diseases. In line with this hypothesis, our docking analysis is supportive of the capacity of BE anthocyanins to directly bind to these transcription factors, this binding could contribute to the observed changes in gene expression following anthocyanin-rich extract supplementation. It should be noted that in addition to significant levels of anthocyanins, BE also contained other polyphenols, including quercetin and phenolic acids, which have been previously reported to exert neuroprotective effects (Khan H. et al., 2019; Szwajgier, Borowiec, & Pustelniak, 2017). Therefore, the potential contribution of other polyphenolic compounds to the observed nutrigenomic effect of BE cannot be ruled out.

Another possible mechanism through which BE could induce the observed nutrigenomic effect is the modulation of miRNAs, the small non-coding RNAs that regulate the gene expression at the post-transcriptional level. The most significant putative miRNA identified in our bioinformatics analysis was miR-124-3p. It is involved in neuronal differentiation, synaptic plasticity, and regulation of learning and memory, with altered expression observed in neurological disorders like AD, PD, and cerebral ischemic stroke (Geng, Liu, & Chen, 2017). Previously, intracerebroventricular administration of a red wine polyphenol resveratrol for one week reduced hippocampal mir-124 levels, which was associated with improved long-term potentiation and memory formation in these mice (Zhao et al., 2013). The identified putative miRNAs also included some associated with dendritogenesis (miR-329-5p and miR-15a-5p) (Zhang et al., 2014), axon guidance (miR-340-5p) (Pietrzykowski & Spijker, 2014), hippocampus-dependent memory formation, blood–brain barrier function, and cognition (miR-181a-5p, miR-9-5p) (Wei et al., 2017; Wu et al., 2019). Recently, anthocyanin gut metabolite, ferulic acid, was shown to reverse spatial memory formation and reduce neuronal death in adult rats exposed to neonatal hypoxic-ischemic brain damage by inhibiting hippocampal miR-9 expression (Yao et al., 2020). To the best of our knowledge, no other studies examined the effect of anthocyanins on the brain expression of any of the top miRNAs identified in our analysis. However, these compounds have been previously reported to modulate miRNAs in other tissues and cells (Krga et al., 2018; Ma & Ning, 2019; Milenkovic et al., 2012). Therefore, by potentially modulating the expression of the aforementioned miRNAs, anthocyanin-rich BE could induce the observed nutrigenomic effects associated with cognitive function and neurodegenerative diseases. However, needless to say that further investigations are needed to confirm the impact of dietary anthocyanins on miRNAs as post-transcriptional regulators of different physiological and pathophysiological processes in the hippocampus.

In conclusion, in the present study, we showed that anthocyanin-rich bilberry extract supplementation could significantly affect the global gene expression in the hippocampus of $ApoE^{-/-}$ mice. The obtained results allow to make assumption about the potential molecular mechanisms underlying the reported neuroprotective effects of anthocyanin-rich food consumption. Bioinformatic analyses suggested that these genes are relevant in the regulation of neuroinflammation, neuronal function, or brain vascular endothelial function, processes that are implicated in cognitive function and neurodegenerative disorders like AD and PD (Fig. 9). Moreover, beyond neurodegenerative disorders, the results suggest that this type of supplementation could also be beneficial in the setting of neuropsychiatric disorders. These results, therefore, provide novel findings of the potential molecular targets and mechanisms of neuroprotective action of anthocyanin-rich food sources. Still, further mechanistic studies validating these mechanisms *in vivo* are warranted.

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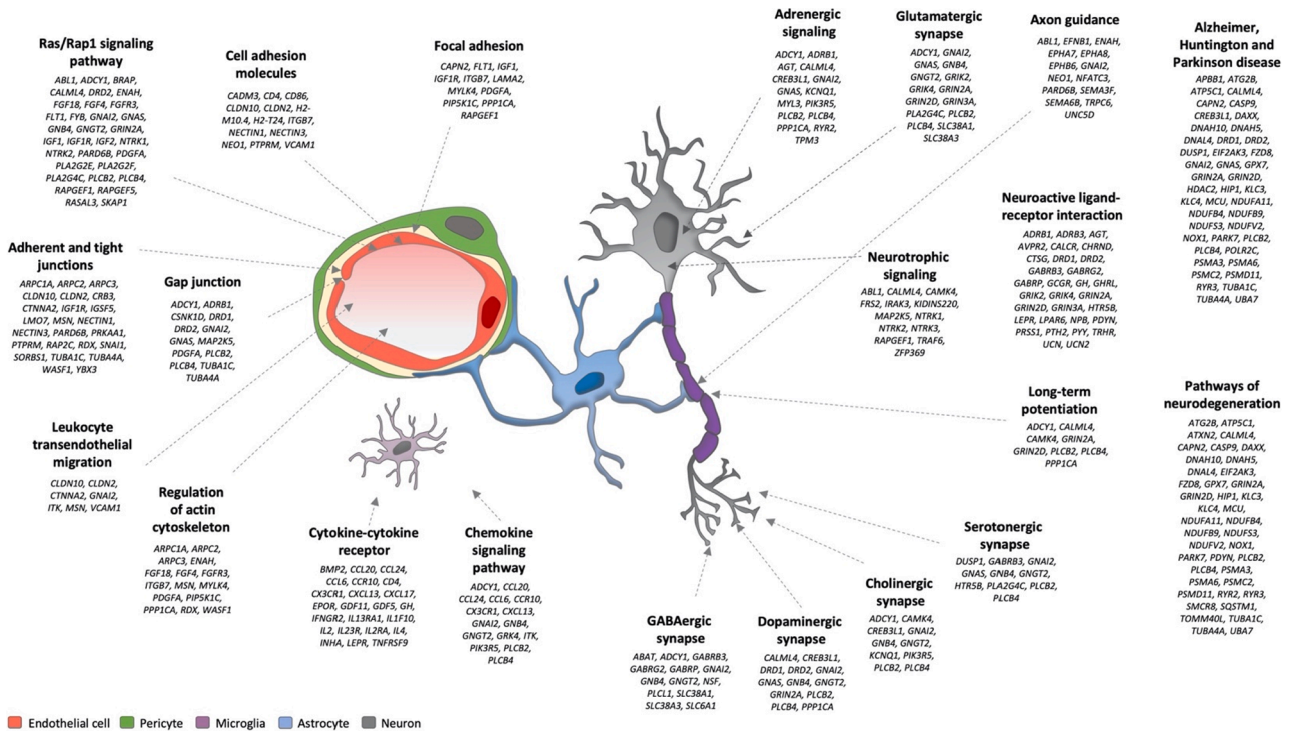

Fig. 9. A summary of the effect of anthocyanin-rich BE supplementation on the expression of genes implicated in neuro-related functions.

CRediT authorship contribution statement

Dragan Milenkovic: Conceptualization, Funding acquisition, Writing - review & editing. **Irena Krga:** Writing - review & editing, Visualization. **Anne-Laure Dinel:** Conceptualization, Data curation, Methodology. **Christine Morand:** Conceptualization, Writing - review & editing. **Sophie Laye:** Conceptualization, Writing - review & editing. **Nathalie Castanon:** Writing - review & editing, Methodology, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2021.104609.

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