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Anthocyanins restore behavioral and biochemical changes caused by streptozotocin-induced sporadic dementia of Alzheimer's type

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ABSTRACT

Aims: The aim of this study was to analyze if the pre-administration of anthocyanin on memory and anxiety prevented the effects caused by intracerebroventricular streptozotocin (icv-STZ) administration-induced sporadic dementia of Alzheimer's type (SDAT) in rats. Moreover, we evaluated whether the levels of nitrite/nitrate (NOx), Na^+,K^+-ATPase, Ca^{2+}-ATPase and acethylcholinesterase (AChE) activities in the cerebral cortex (CC) and hippocampus (HC) are altered in this experimental SDAT.
Main methods: Male Wistar rats were divided in 4 different groups: control (CTRL), anthocyanin (ANT), streptozotocin (STZ) and streptozotocin + anthocyanin (STZ + ANT). After seven days of treatment with ANT (200 mg/kg; oral), the rats were icv-STZ injected (3 mg/kg), and four days later the behavior parameters were performed and the animals submitted to euthanasia.
Key findings: A memory deficit was found in the STZ group, but ANT treatment showed that it prevents this impairment of memory ($P < 0.05$). Our results showed a higher anxiety in the icv-STZ group, but treatment with ANT showed a *per se* effect and prevented the anxiogenic behavior induced by STZ. Our results reveal that the ANT treatment (100 μM) tested displaces the specific binding of [^{3}H] flunitrazepam to the benzodiazepinic site of $GABA_A$ receptors. AChE, Ca^+-ATPase activities and NOx levels were found to be increased in HC and CC in the STZ group, which was attenuated by ANT ($P < 0.05$). STZ decreased Na^+,K^+-ATPase activity and ANT was able to prevent these effects ($P < 0.05$).
Significance: In conclusion, these findings demonstrated that ANT is able to regulate ion pump activity and cholinergic neurotransmission, as well as being able to enhance memory and act as an anxiolytic compound in animals with SDAT.

Introduction

Anthocyanins (ANTs) belong to the flavonoid family, which present phenolic groups in their chemical structure and give colors to a great variety of flowers and fruits (Table 1) (Veitch and Grayer, 2011; Williams and Grayer, 2004; Yoshida et al., 2009). It has been shown that ANTs are potent antioxidants, and are effective scavengers of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Kahkonen and Heinonen, 2003; Kahkonen et al., 2001), having a clear neuroprotective role (Min et al., 2011). There is evidence that ANTs have beneficial effects on memory and cognition (Shukitt-Hale et al., 2009) improving the memory in old rats and humans (Andres-Lacueva et al., 2005; Krikorian et al., 2010).

Acetylcholinesterase (AChE) is an important regulatory enzyme that rapidly hydrolyzes the neurotransmitter acetylcholine (ACh) released by the cholinergic neurons (Paleari et al., 2008). Several experimental and clinical studies clearly indicate an undisputed major role of ACh in the regulation of cognitive functions (Blokland, 1995). Recently, several therapeutic strategies that enhance AChE activity have been implemented to ameliorate cognitive disorders. Cognitive disorders also affect the generation of membrane potentials and the influx of neuronal Ca^{2+} (Berrocal et al., 2009; Mata et al., 2011).

The Na^+,K^+-ATPase and the Ca^{2+}-ATPase are key enzymes in the maintenance of electrolyte gradients in excitable cells and neurons (Jimenez et al., 2010; Panayiotidis et al., 2010). The former enzyme is responsible for the active transport of Na^+ and K^+, and it is necessary

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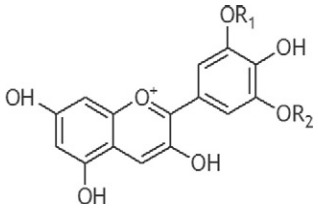
Table 1
Structural identification of anthocyanins.



Anthocyanins	R1	R2	Formula	M.W.
Cyanidin	OH	H	$C_{15}H_{11}O_6$	322.72
Malvidin	OCH_3	H	$C_{16}H_{13}O_6$	336.74
Delphinidin	OH	OH	$C_{15}H_{11}O_7$	338.72
Petunidin	OCH_3	OH	$C_{16}H_{13}O_7$	352.74
Malvidin	OCH_3	OCH_3	$C_{17}H_{15}O_7$	366.77

to maintain the ionic gradient across membranes and thus it is essential to regulate neuronal excitability (Jimenez et al., 2010; Jorgensen et al., 2003; Kaplan, 2002). The Ca^{2+}-ATPase is one of the most powerful modulators of intracellular Ca^{2+} levels (Casteels et al., 1991; Huang et al., 2010; Raeymaekers and Wuytack, 1991). The transient changes in intracellular Ca^{2+} levels regulate a wide variety of cellular processes and cells employ both intracellular and extracellular sources of Ca^{2+} for the activation of signaling pathways and regulation of many physiological and pathological processes (Huang et al., 2010; Missiaen et al., 2000a,b; Ruknudin and Lakatta, 2007).

Alzheimer's disease (AD) is the most common cause of dementia in the elderly, and this disease is characterized by abnormalities in glucose metabolism, reduced glucose utilization and levels of energy rich phosphates (Hoyer, 2004a,b). The intracerebroventricular (icv) injection of STZ in rats has been used as a model of sporadic dementia of AD (Sharma and Gupta, 2001) since it mimics many pathological processes of the disease as impaired brain glucose and energy and leads to progressive deficits in learning and memory (Lannert and Hoyer, 1998).

Considering that AD is the most prevalent neurodegenerative disease worldwide in older adults, we sought to investigate if anthocyanin has the ability to prevent memory deficits induced by icv administration of STZ. We also evaluated the levels of nitrite/nitrate and the activities of enzymes AChE, Na^+,K^+-ATPase and Ca^{2+}-ATPase, which are known to be altered in AD.

Material and methods

Chemicals

Acetylthiocholine, Trizma Base, acetonitrile, Percoll, Coomassie Brilliant Blue G and streptozotocin (STZ) were purchased from Sigma Chemical Co. (St. Luis, MO, USA). Anthocyanins were extracted and purified from grape skin and are commercially available by Christian Hansen A/S. All other reagents used in the experiments were of analytical grade and of the highest purity.

Animals

Male Wistar rats (3 month year old) weighing 350–400 g were used in the study. They were kept in the Central Animal House of the Federal University of Santa Maria in colony cages at an ambient temperature of 25 ± 2 °C and relative humidity 45–55% with 12 h light/dark cycles. They had free access to standard rodent pelleted diet and water *ad libitum*. All procedures were carried out according to the NIH Guide for Care and Use of Laboratory Animals, and the Brazilian Society for Neuroscience and Behavior (SBNeC) recommendations for animal care. This work was approved by the ethical committee of the Federal University of Santa Maria (23081.003601/2012-63).

Administration of drugs to animals

Intracerebroventricular (icv) injection of streptozotocin

Adult male Wistar rats (300–350 g) were anesthetized with thiopental (180 mg/kg). The head was placed in position in the stereotaxic apparatus and a midline sagittal incision was made in the scalp. The stereotaxic coordinates for the lateral ventricle (Paxinos and Watson, 1986) were measured accurately as anterio-posterior −0.8 mm, lateral 1.5 mm and dorso-ventral, −4.0 mm relative to bregma and ventral from dura with the tooth bar set at 0 mm. Through a skull hole, a 28-gauge Hamilton® syringe of 10 μL attached to a stereotaxic apparatus and piston of the syringe was lowered manually into each lateral ventricle. We used 4 different groups: control (CTRL), anthocyanin (ANT), streptozotocin (STZ), and streptozotocin plus anthocyanin (STZ + ANT). The STZ groups received bilateral icv injection of streptozotocin (3 mg/kg, body weight) which was dissolved in citrate buffer (pH 4.4) (Tiwari et al., 2009). The concentration of STZ in citrate buffer was adjusted so as to deliver 5 μL/injection site of the solution. Rats in the control group received icv injection of the same volume of citrate buffer as in the STZ treated (Scheme 1).

Drug administration

Seven to ten animals per group were usually tested in the experiments. Rats were treated by gavage with anthocyanin (200 mg/kg body weight) daily per 7 days (around 10 am). The dose of anthocyanin was chosen on the basis of previous studies indicating neuroprotection (Gutierres et al., 2012b; Manach et al., 2004; Saija et al., 1990; Varadinova et al., 2009). The control groups received only vehicle (2 mL/kg gavage of saline, daily per 7 days).

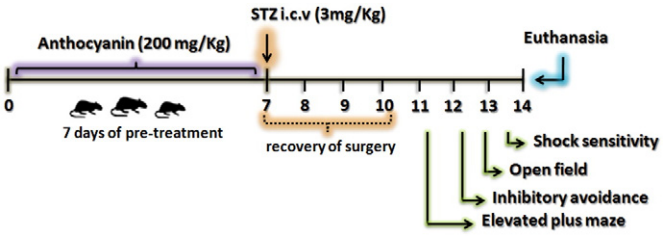
Behavioral procedure

Elevated plus maze task

At the last day of anthocyanin treatment (7th day), the anxiolytic-like behavior was evaluated using the task of the elevated plus maze as previously described (Frussa-Filho et al., 1999; Rubin et al., 2000a). The apparatus consists of a wooden structure raised 50 cm from the floor. This apparatus is composed of 4 arms of the same size, with two closed-arms (walls 40 cm) and two open-arms. Initially, the animals were placed on the central platform of the maze in front of an open arm. The animal had 5 min to explore the apparatus, and the time spent and the number of entries in the open- and closed-arms were recorded. The apparatus was thoroughly cleaned with 30% ethanol between each session.

Inhibitory avoidance task

The animals were subjected to training in a step-down inhibitory avoidance apparatus as previously described (Rubin et al., 2000b). After that the animals received icv-STZ (3 mg/kg). Twenty four hours after the training the animals were subjected to test in a step-down inhibitory avoidance task. Briefly, the rats were subjected to a single



Scheme 1. Exposure design.

training session in a step-down inhibitory avoidance apparatus, which consisted of a 25 × 25 × 35-cm box with a grid floor whose left portion was covered by a 7 × 25-cm platform, 2.5 cm high. The rats were placed gently on the platform facing the rear left corner, and when the rat stepped down with all four paws on the grid, a 3-s 0.4-mA shock was applied to the grid. Retention test took place in the same apparatus 24 h later. Test step-down latency was taken as a measure of retention, and a cut-off time of 300 s was established.

Open field

Immediately after the inhibitory avoidance test session, the animals were transferred to an open-field measuring 56 × 40 × 30 cm, with the floor divided into 12 squares measuring 12 × 12 cm each. The open field session lasted for 5 min and during this time, an observer, who was not aware of the pharmacological treatments, recorded the number of crossing responses and rearing responses manually. This test was carried out to identify motor disabilities, which might influence inhibitory avoidance performance at testing (Gutierres et al., 2012a).

Foot shock sensitivity test

Reactivity to shock was evaluated in the same apparatus used for inhibitory avoidance, except that the platform was removed and was used to determine the flinch and jump thresholds in experimentally naïve animals (Berlese et al., 2005; Rubin et al., 2000a). The animals were placed on the grid and allowed a 3 min habituation period before the start of a series of shocks (1 s) delivered at 10 s intervals. Shock intensities ranged from 0.1 to 0.5 mA in 0.1 mA increments. The adjustments in shock intensity were made in accordance with each animal's response. The intensity was raised by one unit when no response occurred and lowered by one unit when a response was made. A flinch response was defined as withdrawal of one paw from the grid floor, and a jump response was defined as withdrawal of three or four paws. Two measurements of each threshold (flinch and jump) were made, and the mean of each score was calculated for each animal.

Brain tissue preparation

After behavioral tests, the animals were anesthetized under a halothane atmosphere, euthanized by decapitation and the brain was removed and separated into cerebral cortex and hippocampus and placed in a solution of Tris–HCl 10 mM, pH 7.4, on ice. The brain structures were gently homogenized in a glass potter in Tris–HCl solution. Aliquots of resulting brain structure homogenates were stored at −20 °C until utilization (Gutierres et al., 2012a). Protein was determined previously in a strip that varied for each structure: cerebral cortex (0.7 mg/mL) and hippocampus (0.8 mg/mL), and determined by Coomassie blue method as previously described (Bradford, 1976), using bovine serum albumin as standard solution.

Isolation of synaptosomes with a discontinuous Percoll gradient

Synaptosomes were isolated essentially as previously described (Nagy and Delgado-Escueta, 1984), with a minor modification (Gutierres et al., 2012c) using a discontinuous Percoll gradient. The cerebral cortex and hippocampus were gently homogenized in 10 volumes of an ice-cold medium (medium I) containing 320 mM sucrose, 0.1 mM EDTA and 5 mM HEPES, pH 7.5, in a motor driven Teflon-glass homogenizer and then centrifuged at 1000 ×*g* for 10 min. An aliquot of 0.5 mL of the crude mitochondrial pellet was mixed with 4.0 mL of an 8.5% Percoll solution and layered into an isosmotic discontinuous Percoll/sucrose gradient (10%/16%). The synaptosomes that banded at the 10/16% Percoll interface were collected with a wide-tip disposable plastic transfer pipette. The synaptosomal fraction was washed twice with an isosmotic solution consisting of 320 mM sucrose, 5.0 mM HEPES, pH 7.5, and 0.1 mM EDTA by centrifugation at 15,000 ×*g* to remove the contaminating Percoll. The pellet of the second centrifugation was resuspended in an isosmotic solution to a final protein concentration of 0.4–0.6 mg/mL. Synaptosomes were prepared fresh daily and maintained at 0°–4° throughout the procedure and used to measure Ca^{2+}-ATPase and AChE activities.

Assay of lactate dehydrogenase (LDH)

The integrity of the synaptosome preparations was confirmed by the lactate dehydrogenase (LDH) activity, which was obtained after synaptosome lysis with 0.1% Triton X-100 and comparing it with an intact preparation, using the Labtest kit (Labtest, Lagoa Santa, MG, Brazil).

Determination of AChE activity in brain

The AChE enzymatic assay was determined by a modification of the spectrophotometric method (Rocha et al., 1993) as previously described (Ellman et al., 1961). The reaction mixture contained 100 mM K^+-phosphate buffer, pH 7.5 and 1 mM 5,5′-dithiobisnitrobenzoic acid (DTNB). The method is based on the formation of the yellow anion, 5,5′-dithio-bis-acid-nitrobenzoic, measured by absorbance at 412 nm during 2 min incubation at 25 °C. The enzyme (40–50 μg of protein) was pre-incubated for 2 min. The reaction was initiated by adding 0.8 mM acetylthiocholine iodide (AcSCh). All samples were run in triplicate and the enzyme activity was expressed in μmol AcSCh/h/mg of protein.

Analysis of gene expression using semiquantitative RT-PCR

The analysis of AChE expression was carried out using semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR). The hippocampus and cerebral cortex were dissected under sterile conditions, and total RNA was extracted using the TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The RNA was quantified by spectrophotometry, and cDNA was synthesized using the ImProm-II™ Reverse Transcription System (Promega). PCR reactions for the AChE and β-actin genes were performed using 0.1 μM of the appropriate primers (AChE forward: 5′-GAC TGC CTT TAT CTT AAT GTG-3′ and reverse: 5′-CGG CTG ATG AGA GAT TCA TTG-3′; β-actin forward 5′-TAT GCC AAC ACA GTG CTG TCT GG-3′ and reverse 5′-TAC TCC TGC TTC CTG ATC CAC AT-3′) (see Table 2), 0.2 μM dNTP, 2 mM $MgCl_2$, and 0.1 U Platinum Taq DNA polymerase (Invitrogen) in a total volume of 25 μL for AChE and 20 μL for β-actin (Da Silva et al., 2008). The following conditions were used for the PCR reactions: 1 min at 94 °C; 1 min at the annealing temperature (54 °C for β-actin and 55 °C for AChE) and 1 min at 72 °C for 35 cycles. Post-extension at 72 °C was performed for 10 min. For each set of PCR reactions, a negative control was also included. The PCR products (AChE, 785 bp; β-actin, 210 bp) were analyzed on a 1.5% agarose gel containing GelRed® (Biotium) and visualized under ultraviolet light. The Low DNA Mass Ladder (Invitrogen) was used as a molecular marker, and normalization was performed using β-actin as the constitutive gene. All PCR analysis was run in triplicates, including negative controls (in which no reverse transcriptase nor cDNA-containing samples were added in the PCR mix). No background fluorescence was observed when control samples were analyzed (data not shown).

Table 2
The primers used for the gene amplification.

PCR primers design		
Proteins	Primer sequences (50–30)	Accession number (mRNA)
β-Actin[a]	F-TATGCCAACACAGTGCTGTCTGG R-TACTCCTGCTTCCTGATCCACAT	ENSDRT-0000055194
AChE[a]	F-GACTGCCTTTATCTTAATGTG R-CGGCTGATGAGAGATTCATTG	NP_571921

[a] According to da Silva et al. (2008).

Na^+,K^+-ATPase activity measurement

Na^+,K^+-ATPase activity was measured as previously described (Wyse et al., 2000) with minor modifications (Carvalho et al., 2012). Briefly, the assay medium consisted of (in mM) 30 Tris–HCl buffer (pH 7.4), 0.1 EDTA, 50 NaCl, 5 KCl, 6 $MgCl_2$ and 50 μg of protein in the presence or absence of ouabain (1 mM), in a final volume of 350 μL. The reaction was started by the addition of adenosine triphosphate to a final concentration of 3 mM. After 30 min at 37 °C, the reaction was stopped by the addition of 70 μL of 50% (w/v) trichloroacetic acid. Saturating substrate concentrations were used, and reaction was linear with protein and time. Appropriate controls were included in the assays for non-enzymatic hydrolysis of ATP. The amount of inorganic phosphate (Pi) released was quantified colorimetrically, as previously described (Fiske and Subbarow, 1927), using KH_2PO_4 as reference standard. Specific Na^+,K^+-ATPase activity was calculated by subtracting the ouabain-insensitive activity from the overall activity (in the absence of ouabain) and expressed in nmol of Pi/min/mg of protein.

Ca^{2+}-ATPase activity measurement

Ca^{2+}-ATPase activity was measured as previously described (Rohn et al., 1993) with minor modifications (Trevisan et al., 2009). Briefly, the assay medium consisted of (in mM) 30 Tris–HCl buffer (pH 7.4), 0.1 EGTA, 3 $MgCl_2$ and 100 μg of protein in the presence or absence of 0.4 $CaCl_2$, in a final volume of 200 μL. The reaction was started by the addition of adenosine triphosphate to a final concentration of 3 mM. After 60 min at 37 °C, the reaction was stopped by the addition of 70 μL of 50% (w/v) trichloroacetic acid. Saturating substrate concentrations were used, and reaction was linear with protein and time. Appropriate controls were included in the assays for non-enzymatic hydrolysis of ATP. The amount of inorganic phosphate (Pi) released was quantified colorimetrically, as previously described (Fiske and Subbarow, 1927), using KH_2PO_4 as reference standard. The Ca^{2+}-ATPase activity was determined by subtracting the activity measured in the presence of Ca^{2+} from that determined in the absence of Ca^{2+} (no added Ca^{2+} plus 0.1 mM EGTA) and expressed in nmol of Pi/min/mg of protein.

[^{3}H] Flunitrazepam binding assay

To determine if the effect of anthocyanins can be mediated by the $GABA_A$/BDZ complex, we performed a specific binding assay of the BDZ site of $GABA_A$ receptors using [^{3}H] flunitrazepam according to a previous study (Della-Pace et al., 2013). Cerebral cortex from each animal was thawed and homogenized in 10 mL of homogenization buffer A (10 mM Tris–HCl, 300 mM sucrose, and 2 mM EDTA, pH 7.4) per gram of tissue. This homogenate was centrifuged at 1000 ×*g* for 10 min at 4 °C. The resulting supernatant was centrifuged at 16,000 ×*g* for 20 min at 4 °C. The resulting pellet was then resuspended in 1 mL of homogenization buffer and frozen at −70 °C until analyzed.

Radioligand binding assay

[^{3}H] Flunitrazepam binding to the benzodiazepinic site of $GABA_A$ receptors was determined by first washing the cell membrane preparation as follows: individual aliquots were diluted with five volumes of wash buffer B (50 mM Tris–HCl and 2 mM EDTA, pH 7.4), mixed, and centrifuged at 16,000 ×*g* for 10 min at 4 °C, and the samples were incubated for 30 min at 37 °C. This washing procedure was repeated twice, and the final pellet was resuspended in binding assay buffer C (20 mM HEPES and 1 mM EDTA, pH 7.4). The protein concentration of each sample was determined by a spectrophotometric protein dye-binding assay based on the method of Bradford (1976), using bovine serum albumin as the standard. The incubation was carried out in duplicate in polycarbonated tubes (total volume 500 μL) containing 50 mM Tris–HCl (pH 7.4), and 0.5 mg of protein membrane. Diazepam (0.1 μM) was used as a positive control. Incubation was started by adding 1 nM [^{3}H] flunitrazepam (85.8 Ci/mmol), and run in ice for 60 min. The reaction was stopped by vacuum filtration and each filter was washed with 15 mL of cold 10 mM Tris–HCl buffer. Filters were individually placed in polycarbonated tubes and 1 mL of scintillation liquid was added. Radioactivity was determined using a Packard Tri-Carb 2100TR liquid scintillation counter. Non-specific binding was determined by adding 1 μM diazepam to the medium in parallel assays. Specific binding was considered as the difference between total binding and non-specific binding. Results were expressed as percentage of specific binding.

Assay of nitrite plus nitrate (NO_2 plus NO_3)

For NOx determination, an aliquot (200 μL of samples) was homogenized in 200 mM Zn_2SO_4 and acetonitrile (96%, HPLC grade). After, the homogenate was centrifuged at 16,000 ×*g* for 20 min at 4 °C and supernatant was separated for analysis of the NOx content as previously described (Miranda et al., 2001). The resulting pellet was suspended in NaOH (6 M) for protein determination.

Glucose analysis

The glucose levels were measured using standard enzymatic methods from Ortho-Clinical Diagnostics® reagents on the fully automated analyzer (Vitros 950® dry chemistry system; Johnson & Johnson, Rochester, NY, USA).

Statistical analysis

Statistical analysis of training and test step-down latencies was carried out by the Scheirer–Ray–Hare extension of the Kruskal–Wallis test (*nonparametric two-way ANOVA*). The open field, binding assay and foot shock sensitivity was analyzed by *one-way ANOVA* followed by student Newman–Keuls. The other tests were analyzed by *two-way ANOVA*, followed by Tukey test, and considered $P < 0.05$ or $P < 0.001$ as significant difference in all experiments.

Results

Glucose levels

During the complete study there were no differences in body weight and water consumption in all groups (data not shown). There was no significant difference between the mean peripheral glucose levels after 3 mg/kg icv-STZ groups and citrate buffer (pH 4.4) icv injection groups. The mean peripheral glucose levels were 109.36 ± 2.20 for the CTRL group, 102.66 ± 2.13 for the ANT group, 110.44 ± 1.92 for the STZ group and 106.77 ± 2.09 for the STZ + ANT group, respectively, indicating that the dose was subdiabetogenic (Table 3).

Behavioral tests

Anthocyanin prevents the impairment of memory induced by STZ

In this study we used 4 groups of animals: control (CTRL), anthocyanin (ANT), streptozotocin (STZ), and streptozotocin plus anthocyanin

Table 3
Effects of anthocyanin (200 mg/kg) treatment and icv-STZ (3 mg/kg) injection on glucose (mg/dL) levels. Data are reported as means ± S.E.M. with 8–10 rats for group. ANOVA (*one-way*) followed by Tukey test.

Groups	Glucose levels (mmol/L)
CTRL	109.36 ± 2.20
ANT	102.66 ± 2.13
STZ	110.44 ± 1.92
STZ + ANT	106.77 ± 2.09

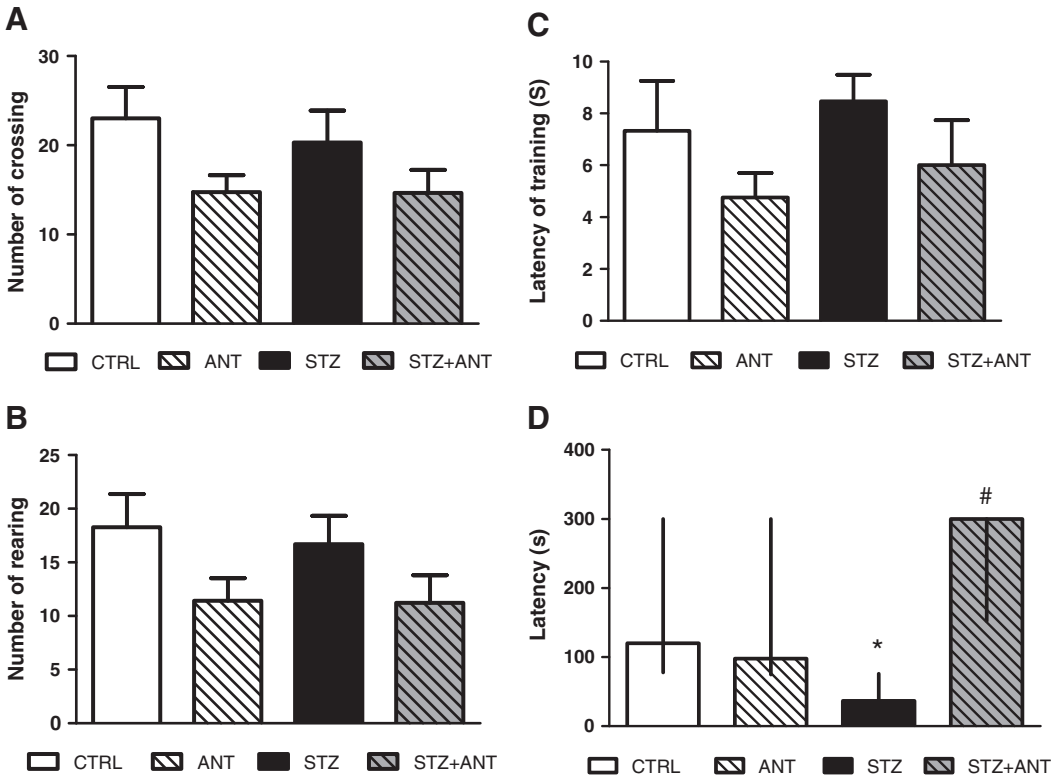


Fig. 1. Oral administration of anthocyanin (200 mg/kg) once a day during 7 days prevents the impairment of memory induced by icv-STZ (3 mg/kg) in adult rats. (A) Number of crossing, (B) number of rearing and (C) latency of training (s) were reported as means ± S.E.M. and analyzed by *one* or *two-way ANOVA*, followed by Tukey test. (D) Latency of test (s) was reported as median ± interquartile range with 8–10 rats for a group. *Denotes $P < 0.05$ as compared to the other groups, # denotes $P < 0.05$ as compared with icv-STZ group by Scheirer–Ray–Hare test (*nonparametric two-way ANOVA*); H2 = 9.75; $P < 0.01$.

(STZ + ANT). Fig. 1 shows the effect of ANT treatment on the STZ-induced memory deficits, in the step-down latencies. Statistical analysis of Scheirer–Ray–Hare test (*nonparametric two-way ANOVA*) showed a significant difference between STZ (3 mg/kg) *vs* ANT (200 mg/kg) or vehicle interaction (CTRL), revealing that treatment with ANT prevented the impairment of memory induced by STZ [H2 = 9.75; $P < 0.01$; Fig. 1D]. Statistical analysis of the data obtained during training showed no difference between the different groups (Fig. 1C).

Although, motivational disparities in the training session may account for differences in inhibitory avoidance testing, experiments were performed to assess whether STZ or ANT affected shock sensitivity threshold and locomotor capacity of the animals. Statistical analysis of open-field data (*one-way ANOVA*) revealed that STZ did not alter the number of crossing ($F_{(3,42)} = 0.11$, $P > 0.05$; Fig. 1A) or rearing ($F_{(3,42)} = 1.82$, $P > 0.05$; Fig. 1B) responses in a subsequent open-field test session, suggesting that neither STZ nor ANT caused gross motor disabilities in this task. Moreover, STZ did not alter foot shock sensitivity, as demonstrated by the similar flinch and jump thresholds exhibited by the animals. In Table 4 it can be seen that neither ANT + STZ animals nor STZ animals were affected in their motor performances and foot shock sensitivity: flinch [$F_{(3,30)} = 1.33$; $P > 0.05$], jump [$F_{(3,30)} = 1.66$; $P > 0.05$] and vocalization [$F_{(3,30)} = 1.76$; $P > 0.05$].

Table 4
Effect of anthocyanin (200 mg/kg) and icv-STZ (3 mg/kg) on foot shock sensitivity (flinch, jump and vocalization). Data are reported as means ± S.E.M. with 8–10 rats for group. ANOVA (*two-way*) followed by Tukey test.

Group	Flinch (mA)	Jump (mA)	Vocalization (mA)
CTRL	0.18 ± 0.01	0.22 ± 0.02	0.42 ± 0.01
ANT	0.20 ± 0.01	0.23 ± 0.02	0.41 ± 0.02
STZ	0.21 ± 0.01	0.21 ± 0.02	0.43 ± 0.01
STZ + ANT	0.18 ± 0.01	0.24 ± 0.01	0.44 ± 0.01
Statistical analysis	$F_{(3,30)} = 1.33$; $p > 0.05$	$F_{(3,30)} = 1.66$; $p > 0.05$	$F_{(3,30)} = 1.76$; $p > 0.05$

Data are means ± S.E.M. for 6–10 animals in each group.

Effect of STZ and anthocyanin treatment on anxiolytic-like behavior

Fig. 2 shows the effect of the treatment with anthocyanin and STZ on anxiolytic-like behavior in the elevated plus maze task. Statistical analysis (*two-way ANOVA*) showed a significant CTRL or STZ (3 mg/kg) *vs* CTRL or ANT (200 mg/kg) interaction to time spent (s) in open arms [$F_{(1,41)} = 6.264$; $P < 0.05$; Fig. 2D] and time in closed arms [$F_{(1,41)} = 4.925$; $P < 0.05$; Fig. 2C], revealing that treatment with ANT prevented the anxiogenic behavior induced by STZ. However, no significant differences in the number of entries in open arms [$F_{(1,41)} = 0.279$; $P > 0.05$; Fig. 2B] and in the number of entries in all arms [$F_{(1,41)} = 0.68$; $P > 0.05$; Fig. 2A] were observed. The number of total entries in arms suggests that neither icv-STZ nor ANT animals had altered locomotor activity in the elevated plus maze task.

Binding of [^{3}H] flunitrazepam to benzodiazepinic site assay

Since we observed an anxiolytic effect of ANT in the elevated plus maze task, we decided to investigate whether the compound can alter the binding of [^{3}H] flunitrazepam to the benzodiazepinic site of a $GABA_A$ receptor. The results presented in Fig. 3 reveal that the ANT (100 μM) reduced by 43% the [^{3}H] flunitrazepam binding to the benzodiazepinic site of $GABA_A$ receptors [$F_{(2,17)} = 47.890$; $P < 0.0001$] and this result demonstrates that ANT can interact with $GABA_A$ receptors.

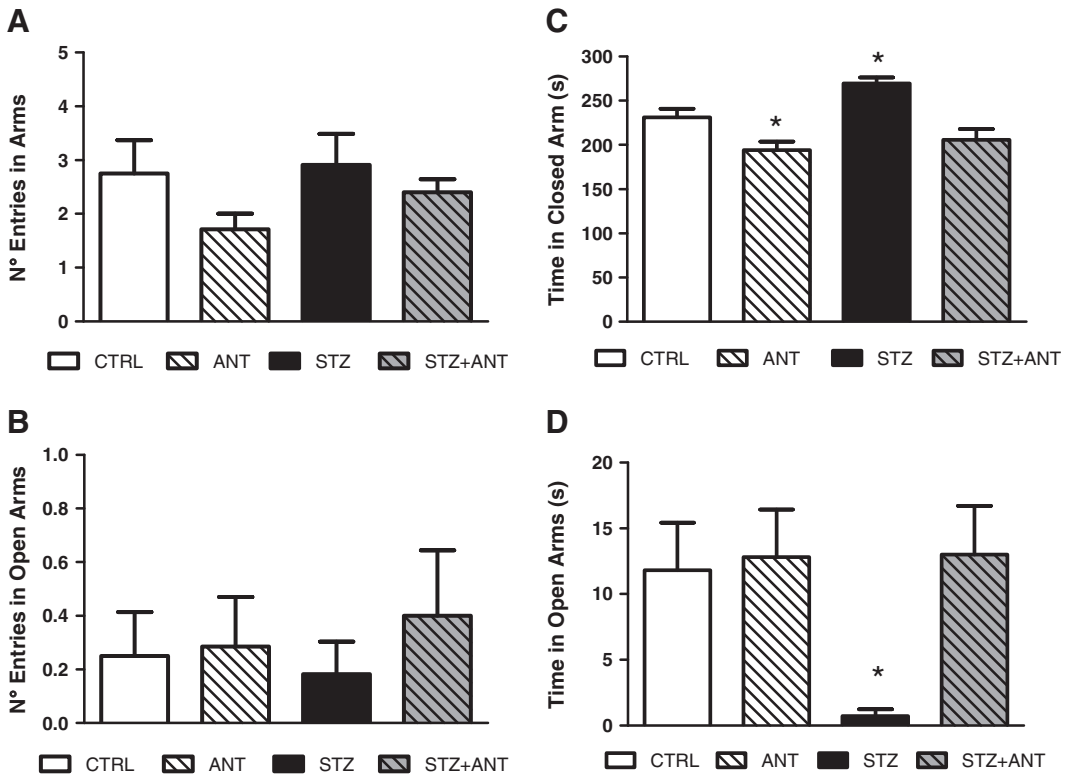


Fig. 2. Effects of anthocyanin (200 mg/kg) treatment and icv-STZ (3 mg/kg) injection on anxiety-like behavior in the elevated plus maze task: (A) number of entries in arms; (B) number of entries in open arms; (C) time in closed arms (s) and (D) percentage of time in open arms. Data are reported as means ± S.E.M. with 8–10 rats for a group. *Denotes $P < 0.05$ as compared to the control (CTRL) group; ANOVA (*two-way*) followed by Tukey test.

Activity and expression of acetylcholinesterase

Anthocyanin prevents the increase in AChE activity induced by STZ

Previous studies report cholinergic impairments in cognitive disorders by quantification of acetylcholinesterase (AChE) activity. Therefore, we investigated whether ANT restores AChE activity in the model of SDAT. Fig. 4 shows the effect of ANT and STZ on the AChE activity in the cerebral cortex and hippocampus, both in S1 and synaptosomes of rats. We found a significant CTRL or STZ (3 mg/kg) *vs* CTRL or ANT (200 mg/kg) interaction, suggesting that the ANT treatment prevents the increase in AChE activity in the S1 fraction of the cerebral cortex [$F = (1,28) = 7.973$; $P < 0.05$] and hippocampus [$F\ (1,28) = 4.995$; $P < 0.05$] (Fig. 4A) induced by icv-STZ.

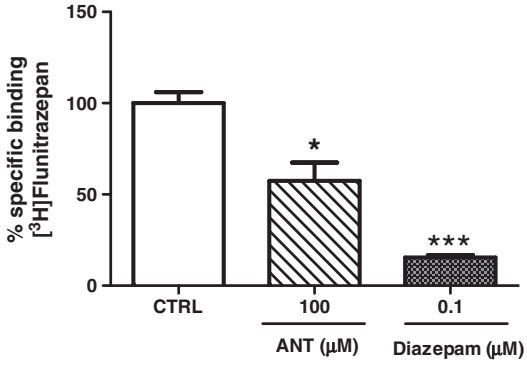


Fig. 3. Anthocyanin (100 μM) reduced the specific [^{3}H] flunitrazepam binding to the benzodiazepinic site of $GABA_A$ receptors. Data are reported as means ± S.E.M. *$P < 0.05$ compared with the diazepan (0.1 μM) and control groups; ***$P < 0.01$ compared with control and ANT groups; ANOVA (*one-way*) followed by Tukey test.

Importantly, synaptosome fraction analysis showed a significant CTRL or STZ (3 mg/kg) *vs* CTRL or ANT (200 mg/kg) interaction, suggesting that the ANT treatment prevents the increase in AChE activity in the synaptosomes of the cerebral cortex [$F\ (1,28) = 4.760$; $P < 0.05$] and hippocampus [$F\ (1,28) = 8.\ 434$; $P < 0.01$](Fig. 4B) induced by icv-STZ.

Effect of STZ and anthocyanin treatment on the AChE expression in the cortex and hippocampus of rats

Fig. 5 shows the effect of ANT and STZ on the AChE expression in the cerebral cortex and hippocampus of rats. No significant differences in AChE expression between groups were observed in the cerebral cortex [$F\ (1,8) = 0.423$; $P > 0.05$] and hippocampus [$F\ (1,8) = 0.140$; $P > 0.05$].

Anthocyanin prevents the decrease of Na^+,K^+-ATPase and increase of Ca^{2+}-ATPase activity induced by STZ

Na^+,K^+-ATPase and Ca^{2+}-ATPase are enzymes involved in the control of neurotransmission, regulating the membrane potential and extracellular calcium concentrations, respectively. Fig. 6 shows the effect of ANT and STZ on the activity of Na^+,K^+-ATPase and Ca^{2+}-ATPase in the cerebral cortex and hippocampus of rats. Statistical analysis (*two-way ANOVA*) showed a significant CTRL or STZ (3 mg/kg) *vs* CTRL or ANT (200 mg/kg) interaction, suggesting that the ANT treatment prevents the decrease in Na^+,K^+-ATPase activity in the cerebral cortex [$F\ (1,28) = 17.760$; $P < 0.001$] and hippocampus [$F\ (1,28) = 4.978$, $P < 0.05$] induced by icv-STZ (Fig. 6A).

Additionally, *two-way ANOVA* showed a significant CTRL or STZ (3 mg/kg) *vs* CTRL or ANT (200 mg/kg) interaction, suggesting that the ANT treatment prevents the increase of Ca^{2+}-ATPase activity in the cerebral cortex [$F\ (1,28) = 5.671$; $P < 0.05$] and hippocampus [$F\ (1,28) = 5.272$; $P < 0.05$] induced by icv-STZ (Fig. 6B).

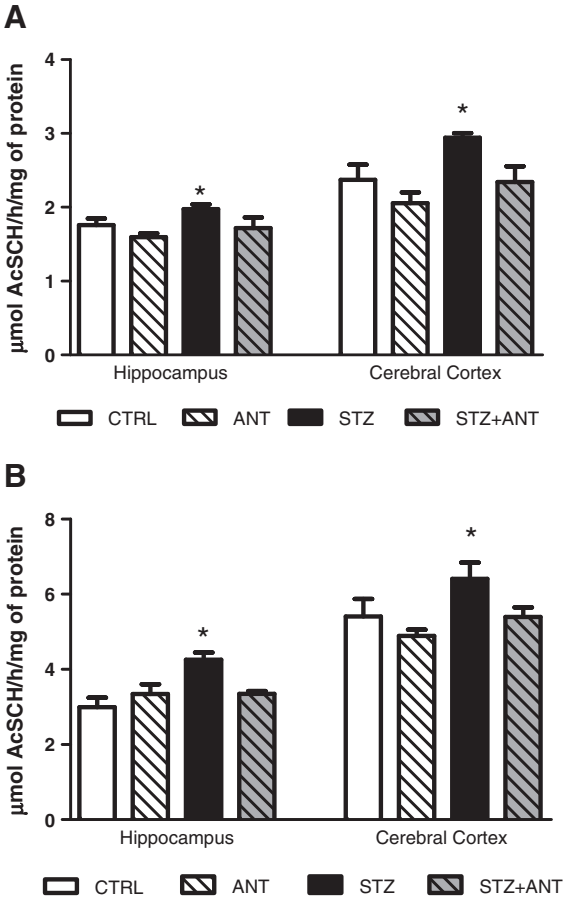


Fig. 4. AChE activity (A) in supernatant fraction and (B) synaptosome fraction of the hippocampus and cerebral cortex in CTRL, ANT, STZ and STZ + ANT groups. Data are reported as means ± S.E.M. with 8–10 rats for a group. *$P < 0.05$ compared with the other groups; ANOVA (*two-way*) followed by Tukey test.

NOx level determination

Anthocyanins are known by their antioxidant properties, so in this set of experiments we investigated if ANT alters nitrite/nitrate content in the brain of rats. Fig. 7 shows the effect of ANT and STZ on the NOx level production in the cerebral cortex and hippocampus. Statistical analysis (*two-way ANOVA*) showed a significant CTRL or STZ (3 mg/kg) *vs* CRTL or ANT (200 mg/kg) interaction, suggesting that the ANT treatment prevents the increased NOx levels both in the cerebral cortex [$F(1,28) = 8.583$; $P < 0.05$] and hippocampus [$F(1,28) = 23.350$; $P < 0.0001$] induced by icv-STZ.

Discussion

Anthocyanins are flavonoids found in fruits and fruit juices, and have the capacity to improve memory (Harborne and Williams, 2001; Williams and Grayer, 2004; Williams et al., 2008). Several evidence have demonstrated that ANTs are able to improve the memory of old rats in Morris water maze (Andres-Lacueva et al., 2005), and of mice in the inhibitory avoidance task (Barros et al., 2006) and elderly humans (Krikorian et al., 2010). There is evidence that ANTs prevent neurotoxicity induced by: i) ethanol in developing brain mice (Ke et al., 2011), ii) reperfusion damage model of cerebral ischemia (Min et al., 2011; Shin et al., 2006), and iii) deleterious effects found in models of Parkinson's (Kim et al., 2010) and Alzheimer's disease (Shih et al., 2010).

Additionally, there was a large number of studies' indicating the neuroprotective role of ANT, since studies have shown that ANT can be transported across biological membranes (Passamonti et al., 2005; Talavera et al., 2005). There are studies announcing gastric absorption and neuroprotective effects of ANT-rich foods, but there is a gap in the knowledge concerning, for example, gastric or ANT transport across the blood–brain barrier (BBB) (Kalt et al., 2008). Another important factor affecting ANT bioavailability and pharmacokinetic properties are their possible ingestion as pigments (anthocyanin derivatives), especially when considering wine consumption. A recent work had already indicated that anthocyanin pyruvic-acid adducts can rapidly reach rat plasma 15 min after oral administration of 400 mg/kg (Faria et al., 2009a,b). Thus, we investigated whether this natural compound could prevent some alterations found in a model of SDAT induced by icv-STZ injection.

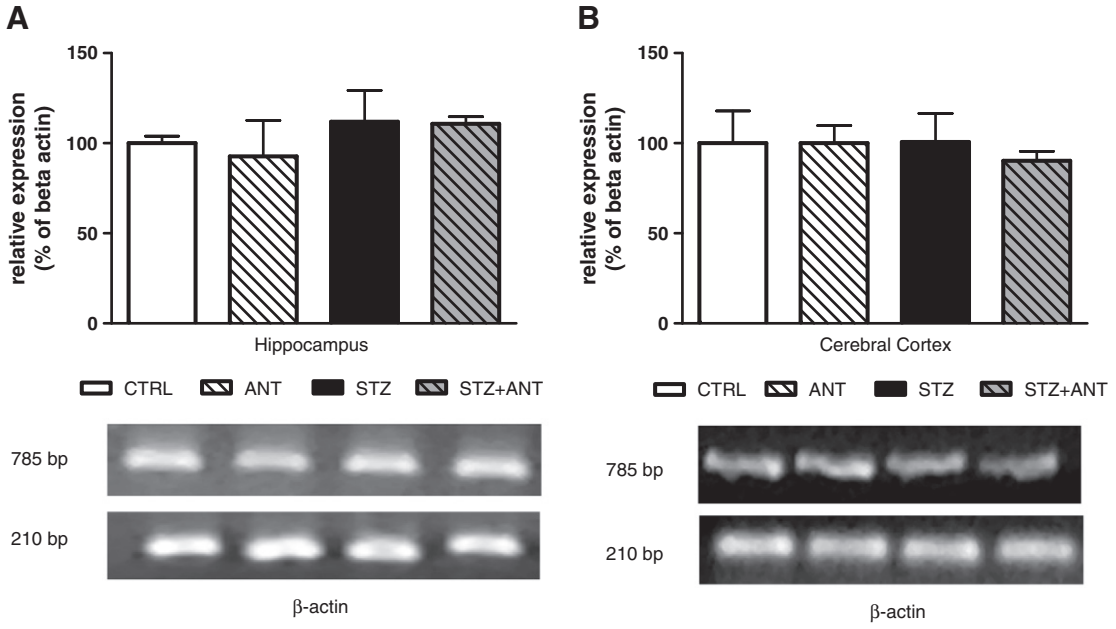


Fig. 5. Relative gene expression pattern of AChE (A) in the hippocampus and (B) cerebral cortex in CTRL, ANT, STZ and STZ + ANT groups. Data are reported as means ± S.E.M. with 4 rats for a group. *$P < 0.05$ compared with the other groups; ANOVA (*two-way*) followed by Tukey test. No significant changes were observed between groups.

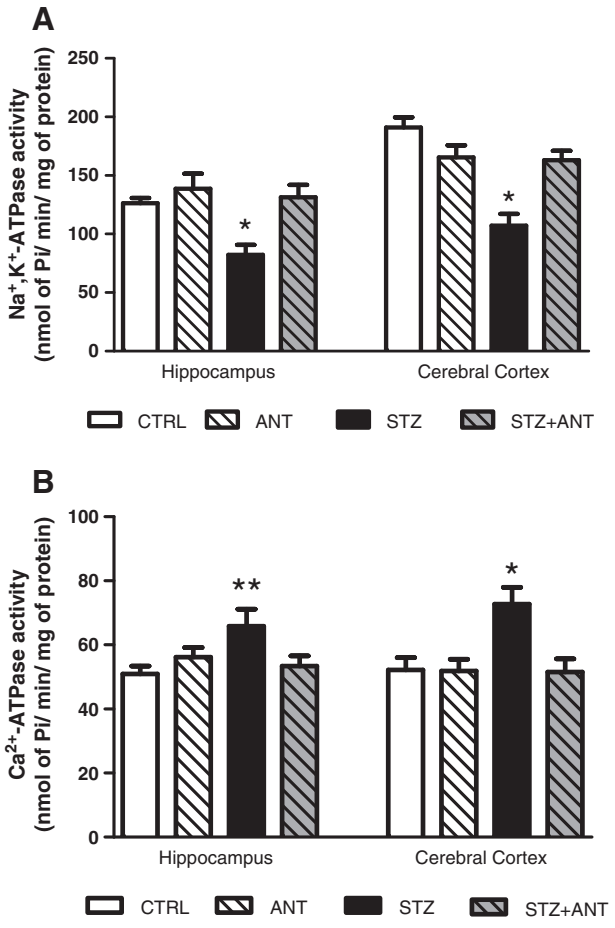


Fig. 6. Na^+,K^+-ATPase (A) and Ca^+-ATPase (B) activity in the hippocampus and cerebral cortex in CTRL, ANT, STZ and STZ + ANT groups. Data are reported as means ± S.E.M. with 8–10 rats for a group. *$P < 0.05$ compared with the other groups; ANOVA (*two-way*) followed by Tukey test.

Furthermore, a subdiabetogenic dose of STZ (3 mg/kg) to rodents causing a progressive memory impairment, loss and synaptic dysfunction (Lannert and Hoyer, 1998; Pinton et al., 2010). Thus, there was no significant difference on the mean peripheral glucose levels between CTRL and icv-STZ groups (Table 3). Our results indicated that icv-STZ impaired the acquisition of memory in rats trained on the inhibitory avoidance task. Interestingly, we found out that ANT at 200 mg/kg for 7 days, did not affect the memory of rats and prevented the memory

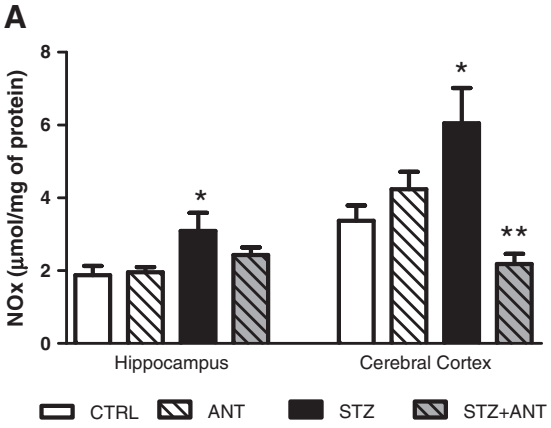


Fig. 7. Effects of anthocyanin and icv-STZ administration on NOx levels in the hippocampus and cerebral cortex of rats. Data are reported as means ± S.E.M. with 8–10 rats for a group. *$P < 0.05$ compared with the other groups; ANOVA (*two-way*) followed by Tukey test.

deficits induced by icv-STZ (Fig. 1D), as assessed by the inhibitory avoidance task. Furthermore, previous studies from our laboratory demonstrated that the dose of 200 mg/kg ANT antagonized scopolamine-induced performance deficits in rats (Gutierres et al., 2012b) suggesting that ANTs have a close interaction with the cholinergic system.

Immediately after inhibitory avoidance test, the animals were subjected to an open-field test which is widely used for evaluating motor abnormalities (Belzung and Griebel, 2001). The open field session revealed that the treatment with icv-STZ or ANT did not alter spontaneous locomotor activity (Fig. 1A, B). Moreover, we observed that the rats of different groups did not show altered shock sensitivity (Table 4), as verified by their similar flinch, jump and vocalization thresholds. These data suggest that neither STZ nor ANT administration caused motor disabilities or altered foot shock sensitivity, excluding their possibility of interference in step-down latencies of inhibitory avoidance task.

Besides assessing the acquisition memory in the inhibitory avoidance task, we also measured the anxiolytic-like behavior by the elevated plus maze task, commonly used to study anxiety-related behavior in rodents (Belzung and Griebel, 2001). Our results showed a higher anxiety in the icv-STZ group (3 mg/kg) (Fig. 2C; D) which is in accordance with previous observations that mice subjected to icv-STZ, in short time (7 days) and long time (21 days) treatments, have an increase in anxious behavior (Pinton et al., 2011). We found an anxiolytic effect of the treatment of ANT that was observed by time in closed arms. However, ANT did not change the number of entries and time spent in open arms. We suggest that investigating anxiolytic effects of anthocyanins *per se* would be important in the selection of a range of doses. Additionally, the dose chosen in this study was able to prevent the anxiogenic behavior caused by icv-STZ administration (Fig. 2C, D). We believe that the mechanism by which ANT plays an anxiolytic effect results in part from an interaction with the GABAergic system, because ANT significantly displaces the specific binding of [^{3}H] flunitrazepam to the benzodiazepinic site of the $GABA_A$ receptor (Fig. 3). Several molecular interactions can be addressed to explain the displacement of the specific binding of [^{3}H] flunitrazepam to the benzodiazepinic site of the $GABA_A$ including the indirect effect of ANT on GABA receptor currents and the binding of ANT at orthosteric or allosteric GABAA binding sites. So, even if we can not assert where ANT is specifically binding, it is clear that it is closely related to the GABAergic pathway. This work is the first to describe a possible location where this compound may act to promote an anxiolytic effect, suggesting that ANT may be considered an important pharmacological agent in situations of anxiety.

The pivotal role of the cholinergic system in memory is further underlined by the use of AChE inhibitors in AD to prevent memory decline. In this study, we found that an icv-STZ group showed an increase in AChE activity in supernatant and synaptosomes in relation to all tested groups (Fig. 4). This finding is in conformity with the previous studies showing an increase in AChE activity upon icv-STZ administration (Awasthi et al., 2010; Tota et al., 2009, 2010), but not AChE expression (Fig. 5). However, we cannot exclude that the icv-STZ injection can cause changes in the AChE mRNA expression, since studies with the administration of icv-STZ for 21 days was able to alter AChE expression in the cerebral cortex and hippocampus of rats (Tota et al., 2012) and the exposure to STZ in this study was 7 days. In addition, the impairment in insulin signaling, reduced cholineacetyltransferase (ChAT) activity and increased oxidative stress induced by icv-STZ injection were associated with the upregulation of AChE in the brain of rats (de la Monte et al., 2006; Lester-Coll et al., 2006). In the present study, we observed that ANT was able to prevent the AChE upregulation in the hippocampus and cerebral cortex of icv-STZ animals, without affecting *per se* the AChE activity. This effect of ANT can be attributed, at least in part, to its potent antioxidant effect.

While it is not evaluated whether glial or neuronal Na^+,K^+-ATPase and Ca^{2+}-ATPAse are preferentially affected by STZ, it is conceivable that STZ may alter both cell types. If this was the case, icv-STZ could alter Na^+, K^+ and Ca^{2+} intracellular gradients, facilitating neuronal

depolarization and impairing sodium and potassium gradient-dependent transport processes, such as neurotransmitter uptake (Benarroch, 2011; Gether et al., 2006; Gouaux, 2009). In this view, it is known that a decreased activity and expression of Na^+,K^+-ATPase, directly affects the signaling of neurotransmitters, impairing learning and memory, as well as locomotor activity and anxiety behavior of rats (dos Reis et al., 2002; Lingrel et al., 2007; Moseley et al., 2007). *In vitro* studies showed that the inhibitor of Na^+,K^+-ATPase, ouabain, increases the Ca^{2+} influx into slices of rat brain (Fujisawa et al., 1965), induces the release of glutamate by reverse transport of Na^+ (Li and Stys, 2001) and cause excitotoxicity in hippocampal neurons (Lees et al., 1990). Corroborating these findings, our study showed that icv-STZ administration decreased Na^+,K^+-ATPase activity and increased Ca^{2+}-ATPase activity (Fig. 6), suggesting that a disturbance in the electrolytic concentrations of Na^+ and Ca^{2+} could lead to excitotoxicity and neuronal death in icv-STZ injected animals.

Furthermore, it was also found that the inhibition of Na^+,K^+-ATPase increases NMDA-mediated currents in the hippocampus (Zhang et al., 2012). It is known that NMDA receptor activation increases the nitric oxide (NO) synthesis by increasing nitric oxide synthase activity (NOS) (Prast and Philippu, 2001; Sattler et al., 1999). NO is a retrograde messenger which diffuses through the cellular membranes and activation of guanylate cyclase and PKG (East and Garthwaite, 1991). Previous studies have demonstrated that activation of NOS and synthesis of NO are related with the reduction of Na^+,K^+-ATPase activity (Boldyrev et al., 2003, 2004; Carvalho et al., 2012). Our results, show that icv-STZ administration increases the nitrate/nitrite levels (Fig. 7), so these findings may be related to the reduction of Na^+,K^+-ATPase in two ways: 1—NO can inhibit the Na^+,K^+-ATPase activity through its binding to thiol groups, generating S-nitrosothiol and consequently leading to the formation of nitrous compounds (Boldyrev and Bulygina, 1997; Boldyrev et al., 1997; Lipton et al., 1993, 1994; Takeguchi et al., 1976); 2—activation of a signaling pathway related with NOS/cGMP/PKG (Carvalho et al., 2012).

These studies have stated that in addition to ANT antioxidant effects, these compounds decrease the levels of NO (Blokland and Jolles, 1993; Juranic and Zizak, 2005). The data presented in this paper demonstrates that ANT prevented the augmentation of NOx levels induced by icv-STZ. Previous studies have shown that ANT is able to decrease the iNOS expression as well as NO production in macrophages and JC77 cells exposed to lipopolysaccharide induced inflammation (Pergola et al., 2006; Wang et al., 2008). This leads us to believe that the ANT might prevent excitotoxic mechanisms related with NO synthesis, since the overproduction of reactive nitrogen species (RNS) results in "nitrosative" stress that contributes to several pathological processes that underlie neurodegenerative and inflammatory diseases (Rutkowski et al., 2007; Valko et al., 2007).

Besides ANT antioxidant properties we can not discard other ANT neuroprotective mechanisms in the prevention of the increase in NOx induced by STZ such as for the affinity of ANT to $GABA_A$ receptors. Studies have shown that compounds which potentiate $GABA_A$ receptors (benzodiazepines) prevent the increase of NO induced by NMDA administration in the cerebellum of rats (Fedele et al., 2000). Furthermore, the activation of $GABA_A$ receptors protects neurons against Aβ toxicity in AD-affected regions in mammalian brain (Paula-Lima et al., 2005). Recent studies have found a significant reduction of GABA currents in AD brains, associated with reductions of mRNA and protein of the principal GABA receptor subunits normally present in the temporal cortex, and these findings can support a functional remodeling of GABAergic neurotransmission in the human AD brain (Limon et al., 2012).

Thus, our results suggest that the ANT could exert beneficial actions, preventing the increase in AChE activity and memory loss induced by icv-STZ. Interestingly, our results showed, for the first time that ANT has affinity for $GABA_A$ receptors, which may explain the anxiolytic effect *per se* and counteract the increased anxiety of icv-STZ animals. Moreover, additional therapeutic implications can be attributed to ANT through its capacity to modulate NO production and regulate Na^+,K^+-ATPase and Ca^+-ATPase activities in pathological situations. More experiments are already being conducted to investigate possible biochemical targets of flavonoids, as ANT, in the SDAT.

Conflict of interest statement

There are no conflicts of interest.

Acknowledgments

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