UNIVERSIDADE FEDERAL DE SANTA MARIA
CENTRO DE CIÊNCIAS NATURAIS E EXATAS
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS
BIOQUÍMICA TOXICOLÓGICA

ESTUDO DA INTERAÇÃO DO SISTEMA PURINÉRGICO, COLINÉRGICO E FUNÇÕES COGNITIVAS NA DEMÊNCIA ESPORÁDICA DO TIPO ALZHEIMER: OS EFEITOS DAS ANTOCIANINAS[1].

TESE DE DOUTORADO

Jessié Martins Gutierres

Santa Maria, RS, Brasil.

2013

[1] O trabalho experimental que constitui a presente tese foi realizado em Santa Maria, Departamento de Química da Universidade Federal de Santa Maria e no Centro de Neurociências e Biologia Celular da Universidade de Coimbra, cidade de Coimbra, Portugal.

ESTUDO DA INTERAÇÃO DO SISTEMA PURINÉRGICO, COLINÉRGICO E FUNÇÕES COGNITIVAS NA DEMÊNCIA ESPORÁDICA DO TIPO ALZHEIMER: OS EFEITOS DAS ANTOCIANINAS

Jessié Martins Gutierres

Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica da Universidade Federal de Santa Maria (UFSM, RS), como requisito para a obtenção do grau de **Doutor em Bioquímica Toxicológica.**

Orientadora: Roselia Maria Spanevello

Co-orientadora: Maria Rosa Chitolina Schetinger

Santa Maria, RS, Brasil.

2013

Ficha catalográfica elaborada através do Programa de Geração Automática
da Biblioteca Central da UFSM, com os dados fornecidos pelo(a) autor(a).

Gutierres, Jessié Martins
Estudo da interação do sistema purinérgico, colinérgico e funções cognitivas na demência esporádica do tipo alzheimer: os efeitos das antocianinas / Jessié Martins Gutierres.-2013.
212 p.; 30cm

Orientadora: Roselia Maria Spanevello
Coorientadora: Maria Rosa Chitolina Schetinger
Tese (doutorado) - Universidade Federal de Santa Maria, Centro de Ciências Naturais e Exatas, Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica, RS, 2013

1. Antocianina 2. Memória 3. Demência 4. Sistema colinergico e purinergico I. Spanevello, Roselia Maria II. Schetinger, Maria Rosa Chitolina III. Título.

E-mail: jessiegutierres@hotmail.com

Centro de Ciências Naturais e Exatas
Universidade Federal de Santa Maria
Programa de Pós-Graduação em
Ciências Biológicas - Bioquímica Toxicológica

A Comissão Examinadora, abaixo-assinada,
aprova a Tese de Doutorado

ESTUDO DA INTERAÇÃO DO SISTEMA PURINÉRGICO, COLINÉRGICO E FUNÇÕES COGNITIVAS NA DEMÊNCIA ESPORÁDICA DO TIPO ALZHEIMER: OS EFEITOS DAS ANTOCIANINAS

elaborada por
Jessié Martins Gutierres

como requisito para obtenção do grau de
Doutor em Bioquímica Toxicológica.

COMISSÃO EXAMINADORA:

_____
Roselia Maria Spanevello, Profª. Drª. (UFPEL)
(Presidente/Orientadora)

_____
Nilda Barbosa, Profª. Drª. (UFSM)

_____
Luiz Fernando Freire Royes, Profº. Dr. (UFSM)

_____
Diogo de Oliveira Losch; Profº. Dr. (UFRGS)

_____
Elisandra Braganhol; Profª. Drª. (UFPEL)

Santa Maria, 27 de março de 2013

scolho os meus amigos, não pela cor da pele ou outro arquétipo qualquer, mas pela pupila dos olhos, Tem que ter um brilho questionador e uma tonalidade inquietante. A mim não interessam os bons de espíritos, nem os maus de hábitos. Fico com os que fazem de mim louco e santo. Deles não quero respostas, quero o meu avesso. Que me tragam dúvidas e angústias e aguentem o que há de pior em mim. Para isto, só sendo louco. Quero os santos, para que não duvidem das diferenças e peçam perdão pelas injustiças. Escolho os meus amigos pela alma lavada e pela cara exposta. Não quero só ombros e o colo, quero também sua maior alegria. Amigo que não ri junto, não sabe sofrer junto. Meus amigos são todos assim, metade maluquice, metade seriedade. Não quero risos previsíveis, nem choros piedosos. Quero amigos sérios que fazem da realidade sua fonte de aprendizagem, e que lutem para que a fantasia não desapareça. Não quero amigos adultos nem chatos. Quero-os metade infância e outra metade velhice! Crianças para que não esqueçam o valor do vento no nosso rosto; e velhos para que nunca tenham pressa. Tenho amigos para saber quem sou. Pois vendo os loucos e santos, bobos e sérios, crianças e velhos nunca me esquecerei de que a 'normalidade' é uma ilusão imbecil e estéril…"

Oscar Wild

Dedico este trabalho.

.... à memória de minha querida avó Camila Nunes Martins, por tudo o que me ensinou, por todo o seu amor e carinho, e por tudo que me deixaste de herança!

.... aos meus queridos país Sonia Martins Gutierres e Geny Cunha Gutierres, por todo o seu amor, zelo, compaixão, carinho e pela educação que sempre me deram, apoiando-me nas horas de aflição, e erguendo-me com a coragem que sempre tiveram para enfrentar os desafios da vida!

Agradecimentos

Desafio tão grande quanto escrever esta Tese, foi utilizar apenas duas páginas para agradecer as pessoas que fizeram parte desta minha trajetória de 8 anos pela Bioquímica da Universidade Federal de Santa Maria, desde a minha iniciação científica até a conclusão do doutorado. Neste período, aprendi que uma tese ou qualquer outro trabalho é uma extensão da vida do autor. Então para que algo de valor seja criado, a pessoa deve primeiro criar algo de valor em si. Por isso, quero agradecer a todas as pessoas que me ajudaram a construir algo de valor em minha vida.

Inicio estes agradecimentos por minha avó, Camila Nunes Martins, por fazer de mim um apaixonado pelos velhos, por dar-me a real dimensão da velhice, por compreender seus passos vacilantes, as suas mãos tremulas e por fazer de mim um ouvinte paciente das histórias repetidas que contava como se fosse à primeira vez. Obrigado minha avó, por fazer de mim uma pessoa melhor e destinar meus esforços para a pesquisa com envelhecimento.

Agradeço aos meus pais, que sempre primaram por minha educação. Obrigado Geny Cunha Gutierres e Sonia Martins Gutierres por, além de me oferecerem a oportunidade de estudar, sempre estiveram ao meu lado nos momentos mais difíceis, este trabalho é fruto de um esforço sobre-humano de vocês, e é também uma homenagem a memória de meu velho pai.

Aos meus irmãos, por estarem sempre torcendo por mim e me incentivando a seguir minhas metas.

Aos meus tios, pelo carinho e incentivo durante todos estes anos.

À minha orientadora Professora Drª. Rosélia Spanevello, uma orientadora exemplar, que sempre respeitou os meus limites e compreendeu as minhas dificuldades, e também investiu no meu potencial, agradeço por toda a imensa ajuda no meu trabalho, paciência e dedicação. Você esteve presente durante toda a minha vida acadêmica, e quero agradecer por ter a oportunidade de ser teu amigo e partilharmos de coisas boas da nossa amizade, você é um exemplo de profissional bem sucedida e de pessoa, e sem dúvida nossa amizade perdurará sempre.

À minha mãe científica e orientadora Professora Drª Maria Rosa Chitolina Schetinger, por me dar a oportunidade de conhecer o mundo científico, valorizando a pesquisa, sempre buscando alinhar o conhecimento científico com a sabedoria, ao seu lado aprendi o que é fazer Ciência, qual a nossa atitude diante do futuro que nos espera, assim levarei para a minha vida toda os seus ensinamentos, sua amizade, dedicação e carinho.

Ao Professor Rodrigo Cunha agradeço imensamente a excelente oportunidade que tive durante o doutorado Sandwich e fazer parte do seu grupo "*Purines at CNC*" no Centro de Neurociências e Biologia Celular de Coimbra, na Universidade de Coimbra. Obrigado pela confiança e pela possibilidade de retornar.

A Professora Paula Agostinho que também pertencente ao "*Purines at CNC*" por compartilhar momentos de aprendizagem e enriquecimento científico em Coimbra. Obrigado por ter sua amizade mesmo estando no outro lado do atlântico.

À Professora Drª Cinthia Mellazzo Mazzanti, que carinhosamente à descrevo como rainha do *Buddha*, minha mãe espiritual, que considero um ser humano incrível e dotado de uma sabedoria sem fim, obrigado por tua amizade, e disponibilidade de me ajudar neste trabalho, pelo apoio e incentivo em todos estes anos.

Aos membros da banca examinadora Professores Elizandra Braganhol, Diogo de Oliveira Losch, Luis Fernando Freire Royes e Nilda Barbosa. As Professoras Vania Loro e Luciane Pereira por terem também lido meu trabalho.

Em especial, ao Fabiano Barbosa Carvalho. Obrigado por compartilhar comigo tua amizade sincera e verdadeira, pelas intermináveis discussões científicas madrugadas a fora, e até mesmo na compilação das referências desta tese pelo desagradável ENDNOT. És mais que um amigo, daqueles que ri e chora junto, e que sempre esteve me apoiando. Este trabalho é fruto também do seu companheirismo, carinho, ensinamentos e colaboração.

Aos meus queridos amigos Juliano, Marília, Fátima, Roberta e Maria do Carmo. Obrigado não somente pela contribuição no trabalho, mas também pela sinceridade, amizade, honestidade, carinho e dedicação nos experimentos e pelo bom humor.

A todos os colegas de laboratório e de outros lugares da UFSM, pela amizade, pelo apoio, pela ajuda e carinho, tenho um profundo sentimento de amizade e respeito por todos! Naia, Lu, Javed, Gustavo, Jucy, Carol, Naiara, Dani, Julia, Eduardo, Pauline, Grazi, a professora Sonia e Neca e a todos os outros, muito obrigado!

A UFSM e ao curso de Doutorado em Bioquímica Toxicológica, pela oportunidade e aos professores do programa.

À CAPES pela bolsa concedida e ao Governo Federal.

A todos os amigos e pessoas que de alguma forma me ajudaram, meus sinceros agradecimentos a todos.

RESUMO

Tese de Doutorado Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica Universidade Federal de Santa Maria, RS, Brasil

ESTUDO DA INTERAÇÃO DO SISTEMA PURINÉRGICO, COLINÉRGICO E FUNÇÕES COGNITIVAS NA DEMÊNCIA ESPORÁDICA DO TIPO ALZHEIMER: OS EFEITOS DAS ANTOCIANINAS

Autor: JESSIÉ MARTINS GUTIERRES
Orientadora: ROSELIA MARIA SPANEVELLO
Co-orientador: MARIA ROSA CHITOLINA SCHETINGER
Data e local de Defesa: Santa Maria, 29 de Abril de 2013.

A demência é uma desordem cerebral caracterizada por um declínio em várias funções mentais e resulta em déficits no funcionamento da memória e em uma variedade de tarefas cognitivas. Evidências têm surgerido que as antocianinas (ANT) possuem propreidades antioxidantes, vasodilatadoras e neuroprotetoras, sendo capazes de melhorar a memória. O objetivo do nosso trabalho foi investigar o papel das ANT sobre a memória e, relacionar estas mudanças com o sistema colínergico e purinérgico em um modelo de amnésia induzido por escopolamina (SCO), em um modelo de demência esporádica do tipo Alzheimer (DAS), induzido por administração intracerebroventricular de streptozotocina (ICV-STZ). Foram utilizados ratos machos adultos, os quais foram tratados previamente com um extrado rico em ANT (200mg/kg) durante sete dias. Para cada protocolo de estudo, foi utilizada a mesma dose de ANT variando apenas o tempo de administração das drogas SCO ip (1mg/kg) e ICV-STZ (3mg/kg) especificas para aos modelos de amnésia e de demência, respectivamente. Os resultados obtidos demonstraram que o tratamento prévio de ANT reverteu déficits de memória no modelo de amnésia e no modelo de DAS. Em relação à atividade da enzima AChE foi observado um aumento significativo nos grupos SCO acompanhado por uma diminuição da atividade das enzimas Na^+,K^+-ATPase e Ca^{2+}-ATPase, e estes efeitos foram prevenidos pelo tratamento com ANT. Foi também observado aumento na atividade da NTPDase, diminuição na atividade da ADA e 5'-NT no cérebro de animais injetados com SCO, acompanhado por uma redução nos niveis de ATP e adenosina, ao qual pode comprometer a sinalização purinérgica. E o tratamento com ANT impediu alterações na atividade destas enzimas. Os animais ICV-STZ tratados apresentaram elevado comportamento ansiogênico e o tratamento com ANT previniu este efeito, indicando que as ANT podem ser consideradas moléculas com propriedades ansiolíticas, uma vez que experimentos *in vitro*, mostraram sua afinidade pelo sítio benzodiazepínico de receptores $GABA_A$. Os animais tratados com ICV-STZ apresentaram marcante elevação na atividade da AChE e Ca^{2+}-ATPase, e redução na atividade da Na^+,K^+-ATPase, causando perturbações nas concentrações eletrolíticas de Na^+ e de Ca^{2+} e isto poderia levar a excitotoxicidade neuronal e aumento na atividade da AChE na DAS, e estes efeitos foram prevenidos pela administração de ANT. Além disso, tanto a densidade como a immunorreatividade para a 5'-NT foi marcadamente reduzida em animais ICV-STZ em comparação com o grupo de ratos normais. Além disso, o tratamento com STZ mostrou uma diminuição significativa para a atividade da NTPDase e 5'-NT no modelo em animais ICV-STZ tratados. No hipocampo de animais ICV-STZ tratados foi encontrado uma elevação considerável nos níveis de ROS total, MDA e NOx, indicando marcante estresse oxidativo, que poderia levar a diminuição na viabilidade de neurônios e na atividade das enzimas NTPDase e 5'-NT, uma vez que elas estão ligadas a membrana celular. Neste contexto, podemos sugerir que tanto o sistema colinérgico como purinérgico podem ser regulados pelo tratamento com antocianinas, definindo assim, o uso deste composto como uma nova estratégia para controlar a deteriorização mnemônica no envelhecimento associado a esses dos sistemas.

Palavras-chave: Antocianina. Memória. Demência. Sistema colinergico e purinergico.

ABSTRACT

Thesis of Doctor's Degree Post-Graduate Program in Biological Sciences:
Toxicological Biochemistry Federal University of Santa Maria, RS, Brazil

STUDY OF PURINERGIC AND CHOLINERGIC SYSTEM AND COGNITIVE FUNCTIONS INTERACTION IN SPORADIC DEMENTIA OF ALZHEIMER TYPE: EFFECTS OF ANTHOCYANINS

Author: JESSIÉ MARITNS GUTIERRES
Adviser: ROSELIA MARIA SPANEVELLO
Co-adviser: MARIA ROSA CHITOLINA SCHETINGER
Date and place of the defense: Santa Maria, April, 29 th, 2013.

Dementia is a brain disorder characterized by decline in several mental functions and results in deficits in memory functioning in a variety of cognitive tasks. Evidence has emerged showing that anthocyanins (ANT) possess antioxidant, vasodilator and neuroprotective properties, and more recently are able to improve memory. The aim of our study was to investigate the role of ANT on memory and relate these changes to the cholinergic and purinergic in a model of amnesia induced by scopolamine (SCO), in a model of sporadic dementia of the Alzheimer type (SDAT), induced by intracerebroventricular injection of streptozotocin (ICV-STZ). We used male adult rats, which were previously treated with an extract rich in ANT (200mg/kg) for seven days. For each study protocol, we used the same dose of ANT only varying the time of drug administration SCO ip (1mg/kg) and ICV-STZ (3mg/kg) specific to the models of amnesia and dementia, respectively. The results showed that pretreatment with ANT reversed the memory deficits in an amnesia model and also in the model of SDAT. Regarding the activity of AChE enzyme was observed a significant increase in SCO group accompanied by a decrease in the activity of Na^+, K^+-ATPase and Ca^{2+}-ATPase, and these effects were prevented by ANT treatment. It was also observed an increase in NTPDase activity, decreased in ADA and 5'-NT activity from brain of animals injected with SCO, accompanied by a reduction in the ATP and adenosine levels, which can compromise the purinergic signaling. ANT treatment was able to prevent changes in NTPDase, ADA and 5'-NT. The ICV-STZ treated animals showed a high anxiogenic-like behavior and ANT treatment prevented this effect, indicating that it can be seen with anxiolytic properties molecules, as *in vitro* experiments demonstrated their affinity for the benzodiazepine site of $GABA_A$ receptors. The animals ICV-STZ treated showed marked increase in AChE activity and Ca^{2+}-ATPase, and decreased activity of Na^+, K^+-ATPase, causing disturbances in electrolyte concentrations of Na^+ and Ca^{2+} and this could lead to neuronal excitotoxic and increased activity AChE in SDAT, and these effects were prevented by administration of ANT. Moreover, both the density and immunoreactivity for 5'-NT was markedly reduced in animals ICV-STZ as compared with the group of -normal rats. In addition, ICV-STZ treatment showed a significant decrease in 5'-NT and NTPDase activity in a model of SDAT. In the hippocampus of ICV-STZ animals was found considerable increase in levels of ROS total, MDA and NOx, indicating marked oxidative stress, which could lead to a decrease in neuronal viability and NTPDase and 5'-NT activity, since they are linked to the cell membrane. In this context, we suggest that both cholinergic and purinergic systems can be regulated by ANT treatment, thus defining use of this compound as a new strategy to control the deterioration of memory in aging associated with these systems.

Keywords: Anthocyanin. Memory. Dementia. Cholinergic and purinergic system.

LISTA DE FIGURAS

Introdução

Figure 1. ***Pomona*** a Deusa das Frutas e do florescimento dos pomares (esquerda) pintura do artista Nicolas Founché (c. 1700) e ***Baco*** o Deus do Vinho, dos excessos e da natureza (c.1595) é uma pintura do barroco italiano de Michelangelo Merisi da Caravaggio (retirado do livro Caravaggio, de Gilles Lambert; Editora Taschen, 2010) (Lambert, 2012) 32

Figura 2. Estrutura das principais classes de flavonóides (CERQUEIRA et al., 2007). 33

Figura 3. Flavonoides induzem em células neuronais e gliais a ativação e inibição de vias de sinalização e de funcionalidade. Adaptado de (Williams e Spencer, 2012).....39

Figura 4. Fotografias de microscopia eletrônica de neurônios de sujeitos normais e com doença de Alzheimer (DA). (a) neurônios normais de sujeito controle; (b) Degeneração neural contendo emaranhados neurofibrilares (setas) em pacientes com DA; (c) Degeneração neuronal mostrando a deposição de peptídeo β-amilóide e acumulação de vacúolos autofágicos na DA. Retirado do artigo de revisão (Yamashima, 2013) 42

Figura. 5. Mecanismo central de ação da estreptozotocina intracerebroventricular administrado em ratos (Mehan, Arora *et al.*, 2012). 45

Figura 6. Representação da Neurotransmissão colinérgica adaptado de (Jones, Byun *et al.*, 2012). 47

Figura 7. Representação do receptor muscarínico metabotrópico (receptor M1), acoplado a uma proteína G, e as vias de sinalização induzidas pela ativação do receptor por seu agonista especifico (Rang, Dale *et al.*, 2001). 48

Figura 8. Receptores isolados e estaticamente espalhados na membrana extracelular (painel superior, adaptado de *H. Matisse, La Musique, 1910, tela a óleo*), em seguida, se agrupam em um "bloco receptor", depois da ativação pelo seu ligante específico o ATP, e a transmissão da sensação de liberação funcional em um sistema (painel inferior, adaptado de *H. Matisse, La Musique, 1910, tela a óleo*) (Volonte, Amadio *et al.*, 2008) 50

Figura 9- Receptores purinérgicos ligam o ATP extracelular e os produtos das reações que resultam da hidrólise enzimática do ATP por ectonucleotidases. Adaptado de (Fields e Burnstock, 2006). 51

Figura 10. Funções do ATP liberado no terminal nervoso e sua completa hidrólise até adenosina no espaço extracelular (Zimmermann, 2001). 52

Figura 11. Atividade catalítica e topografia de membrana para a família das ectonucleotidases Adaptado de (Zimmermann, 2001). 53

Figura 12. Membros da Família das NTPDases. 54

Figura 13. Esquema de eventos celulares relacionados com o envelhecimento cerebral e doenças neurodegenerativas. Adaptado de (Zaidi, 2010) 57

Tabela 1. Identificação estrutural de antocianidinas (agliconas). (Castaneda-Ovando, et al.). *NR: Não reportado..... 35

Sessão Manuscrito I

Scheme 1. *Exposure design*

Table 1 - Effect of anthocyanins treatment (200 mg kg^{-1}) and scopolamine injection (1 mg kg^{-1}) on memory parameters in adult rats. 95

Table 2 - Effect of scopolamine and anthocyanin on the behavior of rats (number of crossing and rearing responses) and on foot shock sensitivity (flinch, jump and vocalization). **..... 96**

Figure 1 - Effect of anthocyanins (200 mg kg^{-1}) and scopolamine (1 mg kg^{-1}) on anxiety-like behavior in adult rats. Bars represent the mean ± SEM. * Represents a significant saline or ANT versus saline or SCO interaction (Two way ANOVA). 97

Figure 2 - Effect of anthocyanins (200 mg kg^{-1}) and scopolamine (1 mg kg^{-1}) on AChE activity in synaptosomes (A) and supernatant (B) in cerebral cortex and hippocampus of rats. Bars represent the mean ± SEM. * Represents a significant saline or ANT versus saline or SCO interaction (Two way ANOVA).... 98

Figure 3 - Effect of anthocyanins (200 mg kg^{-1}) and scopolamine (1 mg kg^{-1}) on Na^+, K^+-ATPase (A) and Ca^{2+}-ATPase (B) activities in cerebral cortex and hippocampus of adult rats. Bars represent the mean ± SEM. * Represents a significant saline or ANT versus saline or SCO interaction (Two way ANOVA).... 99

Figure 4 - Effect of anthocyanins (200 mg kg^{-1}) and scopolamine (1 mg kg^{-1}) on NOx levels in cerebral cortex and hippocampus of rats. Bars represent the mean ± SEM (Two way ANOVA). 100

Sessão Artigo I

Fig. 1. Oral administration of anthocyanins (200 mg kg−1) once a day during 7 days reverses the impairment of memory induced by scopolamine (1 mg kg−1) in adult rats. Data are the median ± nterquartile range for 8–10 animals in each group. *Pb0.05 compared with the other groups, #Pb0.05 compared with the scopolamine (SCO) group by the Scheirer–Ray–Hare nonparametric multiple comparisons task. 106

Fig. 2. Effects of Antho and SCO on NTPDase activity in synaptosomes of the cerebral cortex and hippocampus using ATP (A and B) and ADP (C and D) respectively as substrates. Bars represent the mean±SEM. *Represents a significant difference in all groups, Pb0.05. (ANOVA two-way, Tukey test). 107

Fig. 3. Effects of Antho and SCO on 5′-nucleotidase activity in synaptosomes of the cerebral cortex and hippocampus using AMP as substrate. Bars represent the mean±SEM. *Represents a significant difference in all groups, Pb0.05. (ANOVA two-way, Tukey test). 107

Fig. 4. Effects of Antho and SCO on adenosine deaminase activity in synaptosomes of the cerebral cortex and hippocampus using adenosine as substrate. Bars represent the mean±SEM. *Represents a significant difference in all groups, Pb0.05. (ANOVA two-way, Tukey test). 108

Table 1. Effect of anthocyanins (200 mg kg−1) and scopolamine (1 mg kg−1) on the latency of training, and behavior of rats (number of crossing and rearing responses) in the open-field immediately after the inhibitory avoidance testing session and on foot shock sensitivity (flinch, jump and vocalization). 106

Table 2. Effect of anthocyanins (200 mg kg−1) and scopolamine (1 mg kg−1) on the nucleotides levels (ATP, ADP, AMP and Adenosine) in the cerebral cortex of rats. The data were analyzed by two-way ANOVA followed by Tukey's test and expressed in nmol of nucleotide/mg of tissue. □Represents a significant difference in all groups, Pb0.05. 108

Table 3. Effect of anthocyanins (200 mg kg−1) and scopolamine (1 mg kg−1) on the nucleotides levels (ATP, ADP, AMP and Adenosine) in the hippocampus of rats. The data were analyzed by two-way ANOVA followed by Tukey's test and expressed in nmol of nucleotide/mg of tissue. □Represents a significant difference in all groups, Pb0.05. 108

Sessão Manuscrito II

Figure 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 was 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 group. *Denotes *P<0.05* as compared to the others 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*. 136

Figure 2. Effects of anthocyanin (200mg/kg) treatment and icv-STZ (3mg/kg) injection on anxiety-like behavior in the elevated plus maze task: (A) number of entries in arms; (B) number of entries in open arm; (C) time in closed arms (s) and (D) percentage of time in open arm. Data are reported as means ± S.E.M. with 8-10 rats for group. *Denotes *P<0.05* as compared to the control (CTRL) group, ANOVA (*two-way*) followed by Tukey test. 136

Figure 3. Anthocyanin (100µM) reduced the specific [3H] flunitrazepam binding to 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. 137

Figure 4. AChE activity (A) in supernatant fraction and (B) synaptosomes fraction of 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 group. **P<0.05* compared with the others groups; ANOVA (*two-way*) followed by Tukey test. ... 137

Figure 5. Relative gene expression pattern of AChE (A) in 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 group. *P<0.05 compared with the others groups; ANOVA (two-way) followed by Tukey test. No significative changes were observed between groups. 137

Figure 6. Na^+,K^+-ATPase (A) and Ca^+-ATPase (B) activity in 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 group. **P<0.05* compared with the others groups; ANOVA (*two-way*) followed by Tukey test. 137

Figure 7. Effects of anthocyanin and icv-STZ administration on NOx levels in hippocampus and cerebral cortex of rats. Data are reported as means ± S.E.M. with 8-10 rats for group. **P<0.05* compared with the others groups; ANOVA (*two-way*) followed by Tukey test. 137

Scheme 1. Exposure design

Table 1. Structural identification of anthocyanins.....147

Table 2. The primers used for the gene amplification.....148

Table 3. Effects of anthocyanin (200mg/kg) treatment and icv-STZ (3mg/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.....149

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. 149

Anexo I

Figure 1. Cresyl violet staining in the hippocampal region of control group (A) and ICV-STZ group (B) at 28 days of treatment. In the ICV-STZ group, only a few neurons in CA1 (E), CA3 (F) region are stained compared to CA1 (C) and CA3 (D) region of control group. The dentate gyrus (G) of control group showed many cresyl violet-positive cells are found in compared to dentate gyrus (H) of ICV STZ group. CA1, cornu ammonis 1; CA3, cornu ammonis 3; dentate gyrus. Scale bar=50 µm. 159

Figure 2 Fluoro-Jade C histochemistry staining in hippocampus (CA1, CA3 and giro dentate) after 28 days of the intracerebroventricular STZ or vehicle injections. Digital images of rat brain sections showing no difference for Fluoro-Jade C-positive neurons in hippocampus regions of control (CA1, CA3 and gyro dentate) or ICV-STZ injections (CA1, CA3 and gyro dentate). Original magnification: (A) and (B)×5, (C-H)x20; Scale bar=50 µm. 160

Figure 3. Fluorescence Immunohistochemistry of 5'-nucleotidase (red) and synaptophysin (green) in ICV-STZ and normal brains. Examples of coronal sections of the hippocampus from ICV-STZ and normal brains. 5'-nucleotidase (5'-NT) immunoreactivity in CA3 and dentate gyrus (DG) were observed in normal rat brains, but ICV-STZ group showed lower reactivity for 5'-NT both in CA3 or DG. Similarly, ICV-STZ injection showed weaker immunoreactivity for synaptophysin in CA3 and dentate gyrus compared to the normal brains. Original magnification: (A)×20. 161

Figure 4 - Intracerebroventricular injection of STZ alter proteins density for NTPDase 1 (A) and 5'-NT (B) were determined by Western-blot analysis. Equalization of protein loading was determined using β-actin as the house keeping protein. (C) ATP levels measured by high pressure liquid

chromatography (HPLC). (D) ATPase activity; (E) ADPase activity and (F) 5'NT activity in synaptosomes of hippocampus of CTR and ICV-STZ groups. Data (mean values±SEM) were submitted to t-test statistical analysis. Significance level adopted was *P<0.05 versus CTR group. 162

Figure 5. Intracerebroventricular injection of STZ display changes in cognitive behavior. (A) The CTR groups significantly differ in search times during acquisition training. (B) Probe test after the original training. (C) Number of crossings on the platform. (D) panels plot the % relative time and (E) % of entries in open arms in Elevated plus maze task during the 5-minutes. (F) Novel object recognition assessment of CTR and STZ groups. Recognition index = (time spent exploring a novel object) / (total time spent exploring both novel and familiar objects). # p<0.05; One sample t test relative to CTR (n = 10). 163

Figure 6. Na^+,K^+-ATPase (A) and Ca^+-ATPase (B) activity in hippocampus of intracerebroventricular STZ or vehicle injections. Data (mean values±SEM) were submitted to t-test statistical analysis. Significance level adopted was P<0.05. 164

Figure 7. Effects of intracerebroventricular STZ or vehicle injection on superoxide dismutase activity, MDA, nitrite plus nitrate levels and ROS generation of the hippocampus. Graphs Data (mean values±SEM) were submitted to t-test statistical analysis, (D) Graph express changes in ROS generation (percentage) of STZ group in relation to CTR group. Significance level adopted was P<0.05. 164

LISTA DE ABREVIATURAS

5’-NT, – endo-5’-nucleotidase (E.C. 3.1.3.5)

AKT, – proteína quinase B

ASK-1 – proteína quinase reguladora de sinais apoptóticos-1

A1R – adenosine A1 receptor

A2AR – adenosine A2A receptor

ADA – adenosine deaminase (E.C. 3.5.4.4)

ADP – adenosine 5'-diphosphate

AK – adenosine kinase (E.C. 2.7.1.20)

AMP – adenosine 5'-monophosphate

ANOVA – análise de variância

ANT – antocianinas

ATP – adenosine 5'-triphosphate

Bad e Caspases – proteínas de sinalização pró-apoptótica em neurónios

BHE – barreira hemato encefálica

BDNF – fator neurotrófico derivado do cérebro

BSA – bovine serum albumin

Cy – Cianidina

CREB – proteína ligante ao elemento de resposta do cAMP

cAMP – AMP cíclico

c-JNK1/2/3 – proteínas quinases N-terminas

SNC – Sistema nervoso central

CoA – coenzima A

DA – doença de Alzheimer

DAS – demência esporádica do tipo Alzheimer

Dp – Delfinidina

DMSO – dimetil sulfóxido

EDTA – acido etilenodiaminotetraacético

ERK – Ativação da quinase do receptor extracelular

***et al*.,** – do Latim “*et alii*” ou “*et aliae*”, significando “e outros”

GABA – ácido aminobutírico-γ

GSH – glutationa

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HPLC – cromatografia líquida de alta performance (ou cromatografia líquida de alta pressão)

***i.e.*,** – do Latin "*id est*", significa "que é"

ICV – intracerebroventricular

i.p., – intra-peritonial

LDH – lactato desidrogenase (E.C. 1.1.1.27)

LC-MS/MS – Liquid chromatography–mass spectrometry

LTD – depressão a longo prazo

LTP – potenciação de longo prazo

mTOR – alvo da rapamicina em mamíferos

hinos – óxido nítrico sintase induzível

NO – oxido nítrico

NMDA – *N*-metil-D-aspartato

P1 – tipo 1 de receptores purinérgicos, i.e. receptores de adenosina

P2 – receptors purinérgicos tipo 2, i.e. receptores de nucleotídeos

P2X – Receptor ionotrópico para ATP

P2Y – Receptor metabotrópico de nucleotídeo

PBS – tampão salina-fosfato

PCA – ácido perclórico

PCR – reação da cadeia da polimerase

Pg – Pelargonidina

Pn – Peonidina

Pt – Petunidina

PKB – refere-se a proteína-quinase B

P38 – proteína 38

SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEM – erro padrão da média

STZ – streptozotocina ou 2-deoxy-2-(3-(methyl-3-nitrosoureido)-D-glucopyranose

STAT-1 – proteína de transdução de sinal e ativadora de transcrição da família 1

Tau – taurina

TGF-α – fator de crescimento do tumor α

TBS-T – tampão trizma-salina com Tween-20

VEGF – fator de crescimento endotelial vascular

SUMÁRIO

APRESENTAÇÃO 27

1. INTRODUÇÃO 29

2. OBJETIVOS 59
2.1 Objetivo geral 61
2.2 Objetivos específicos 61

3. MANUSCRITO I 63
3.1 Neuroprotective effects of Anthocyanins on scopolamine-induced amnesia via anti-acetylcholinesterase and membrane bound ATPases in rats 65

4. ARTIGO I 101
4.1. Protective effects of anthocyanins on the ectonucleotidase activity in the impairment of memory induced by scopolamine in adult rats 103

5. MANUSCRITO II 111
5.1 Anthocyanins restore behavioral and biochemical changes caused by streptozotocin-induced sporadic dementia of Alzheimer's type 113

6. ANEXO I 157

7. DISCUSSÃO 167
7.1 Discussão 169

8. CONCLUSÕES 179
8.1 Conclusões 181

REFERÊNCIAS 1877

APRESENTAÇÃO

Os resultados científicos que fazem parte desta tese estão apresentados na forma de artigos originais publicados em revistas e jornais científicos internacionais e manuscritos em fase de preparação, ao qual se encontram no item Manuscrito. As seções Materiais e Métodos, Resultados, Discussão e Referências encontram-se no próprio artigo ou manuscrito e representam a integra deste estudo.

O item Conclusões encontra-se no final desta tese e apresenta interpretações e comentários gerais sobre os resultados contidos neste trabalho.

As referências referem-se somente às citações que aparecem nos itens Introdução, Revisão Bibliográfica e Conclusões desta tese. A seguir encontra-se uma lista com as publicações em revistas e manuscritos em preparação:

1) Jessié M. Gutierres, Fabiano B. Carvalho, Maria Rosa C. Schetinger, Patricia C. Marisco, Marília V. Rodrigues, Cinthia M. Mazzanti, Michele M. Rosa, Juliano M. Vieira, Roberta Schmatz, Maribel A. Rubin, Vera M. Morsch, Roselia Spanevello. **Effect of Anthocyanin on memory retention in rats: Possible involvement of cholinergic system.** Submetido para o periódico: Neurochemical Research.

2) Jessié M. Gutierres, Fabiano B. Carvalho, Maria R.C. Schetinger, Marília V. Rodrigues, Roberta Schmatz, Victor C. Pimentel, Juliano M. Vieira, Michele M. Rosa, Patrícia Marisco, Daniela A. Ribeiro, Claudio Leal, Maribel A. Rubin, Cinthia M. Mazzanti, Roselia Spanevello. **Protective effects of anthocyanins on the ectonucleotidase activity in the impairment of memory induced by scopolamine in adult rats.** Life Sciences 91 (2012) 1221–1228

3) Jessié M. Gutierres, Fabiano B. Carvalho, Paula Agostinho, Maria Rosa C. Schetinger, Patrícia Marisco, Marília Rodrigues, Maribel A. Rubin, Roberta Schmatz, Cassia R. da Silva, Julia C. Farias, Giana de P. Cognato, Michele M. Rosa, Cinthia M. Mazzanti, Mauricio Bogo, Carla D. Bonan, Roselia Spanevello. **Anthocyanins restore behavioral and biochemical changes caused by streptozotocin-induced sporadic dementia of Alzheimer's type**. Em revisão pela Revista Physiology and Behaviour.

4) Anexo I. Resultados complementares, em fase de preparação.

1. Introdução

1 INTRODUÇÃO

Os primeiros estudos dos pigmentos flavonóides foram realizados por Robert Boyle, em 1664, que descreve os efeitos de ácidos e bases sobre a pigmentação e a cor dos extratos de flores de plantas e outros tecidos. Em 1936, o ganhador do Prêmio Nobel Dr. Albert Szent-Gyorgyi e colaboradores relataram que a preparação de flavonóides de páprica e casca de cítricos poderia restaurar a saúde de cobaias com escorbuto, que é provocada pela deficiência de vitamina C da dieta. Szent-Gyorgyi sugeriu que esses pigmentos foram cruciais para a integridade dos vasos sanguíneos e para o tratamento da pele púrpura. Albert Szent-Gyorgyi e colegas tentaram classificar esses compostos vegetais como vitamina P, porém a diversidade química dos flavonóides impede sua classificação como uma vitamina.

Desde a descoberta, os cientistas isolaram mais de 4.000 flavonóides. As isoflavonas estão entre os primeiros flavonóides estudados biologicamente, devido aos grupos hidroxilas nas posições 7 e 4 da estrutura do anel básico, ao qual possui forte afinidade com receptores de estrogénio (Shibata, Shibata *et al.*, 1912).

Os flavonóides compreendem vários tipos de substâncias naturais, entre os quais muitos delas responsáveis pela cor amarela, laranja, vermelha, violeta e azul, de muitas flores, folhas e frutos. Essas substâncias aparentemente desconhecidas da população em geral, estão presentes em grande parte de nossa vida, e também em grande parte na história da humanidade. Na antiguidade a mitologia romana, nomeava deuses para representar a beleza, cor e capacidades nutritivas das frutas, um exemplo é *Pomona* considerada a deusa dos frutos. Seu nome vem da palavra latina *pomum*, que se traduz como "fruto". Ela é deusa unicamente romana, e é particularmente associada com o florescimento das árvores (ver Figura 1). Desde o início da humanidade, mesmo também nos contos bíblicos do paraíso terrestre de Adão e Eva, as frutas de diversas cores, características da presença de flavonóides, estavam simbolizadas na maçã vermelha com que a Eva seduziu Adão. Assim, os flavonóides foram incorporados com certa ironia no pecado original.

E na leitura dos filósofos antigos, torna-se claro que, os flavonóides têm estado presente na mesa de jantar desde os primeiros homens na terra, e servindo também como alimento nutritivo para diversas espécies animais (Shibata, Shibata *et al.*, 1912).

Figure 1. ***Pomona*** a Deusa das Frutas e do florescimento dos pomares (esquerda) pintura do artista Nicolas Founché (c. 1700) e ***Baco*** o Deus do Vinho, dos excessos e da natureza (c.1595) é uma pintura do barroco italiano de Michelangelo Merisi da Caravaggio (retirado do livro Caravaggio, de Gilles Lambert; Editora Taschen, 2010) (Lambert, 2012).

Os flavonóides fenólicos têm sido abundantemente encontrados em sucos de uva e no vinho tinto, e esses compostos possuem diversas propriedades, como antioxidantes, aos quais neutralizam radicais livres e evitam a oxidação de macromoléculas celulares (Waterhouse, 2002). Os compostos fenólicos presentes na uva e seus derivados apresentam uma grande diversidade e são subdivididos em dois grandes grupos em razão da similaridade de suas cadeias de átomos de carbono: flavonóides e não-flavonóides. Do ponto de vista químico, os compostos fenólicos são caracterizados por apresentar um núcleo benzênico, com um ou mais substituintes hidroxílicos, incluindo seus grupos funcionais (Waterhouse, 2002; Abe, 2007). Os flavonóides são caracterizados por um esqueleto base contendo 15 átomos de carbono (C6-C3-C6), do tipo 2-fenil benzopirona. Esta grande família é dividida em inúmeras subclasses, as quais se distinguem entre si através do grau de oxidação do seu grupo

pirano (Bravo, 1998) (Figura 2). Fazem parte deste grupo os flavonóis (catequina, epicatequina e epigalocatequina), flavonas (caempferol, quercetina, rutina e miricetina) e as antocianinas (malvidina e cianidina) (Mamede e Pastore, 2004). Também fazem parte do grupo dos flavonóides, a classe dos dihidroflavonóis e as flavonas das folhas da parreira (Gonzalez-Paramas, Esteban-Ruano *et al.*, 2004).

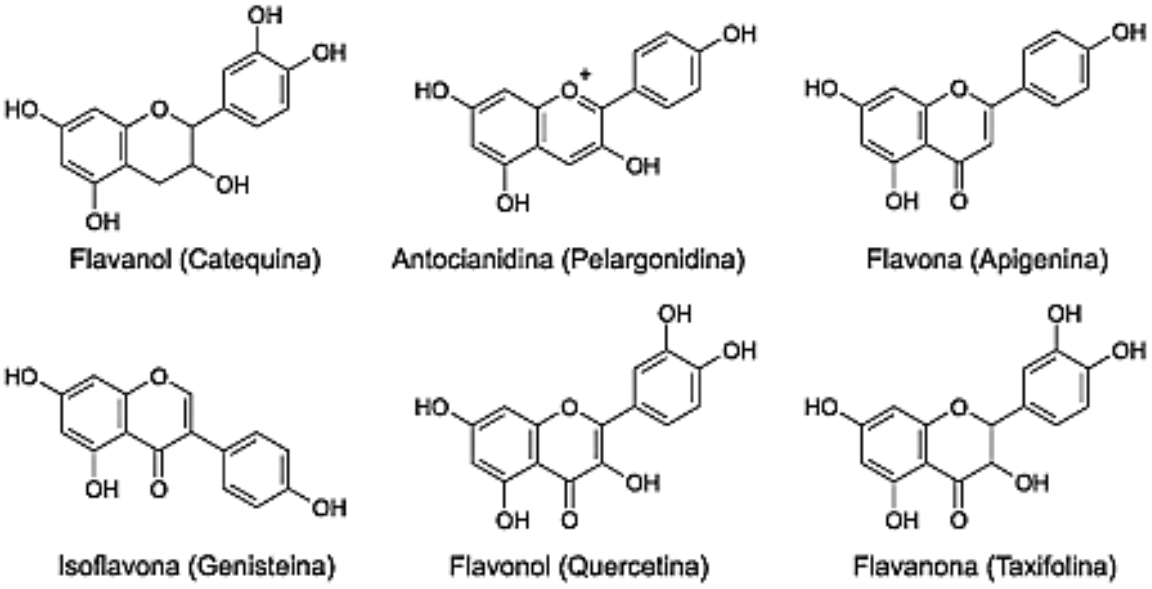


Figura 2. Estrutura das principais classes de flavonóides (CERQUEIRA et al., 2007).

O termo antocianina, derivado das palavras gregas *anthos* (flor) e *kyans* (Azul), foi introduzido em 1835 por Ludwig Clamor Marquart, em seu livro *Die Farben der Bluthen*, para designar substancias azuis extraídas de algumas flores. Atualmente, o termo é empregado para indicar genericamente toda esta família de pigmentos naturais, independentemente da coloração que possam apresentar (Whiting, 2001; Curtright, Emry *et al.*, 2004). O estudo das antocianinas começou na metade do século XIX. A cianina, uma das substancias mais abundantes, foi isolada em 1854, mas sua formula molecular só foi estabelecida em 1913 (Shibata, Shibata *et al.*, 1912; Spencer, 2009).

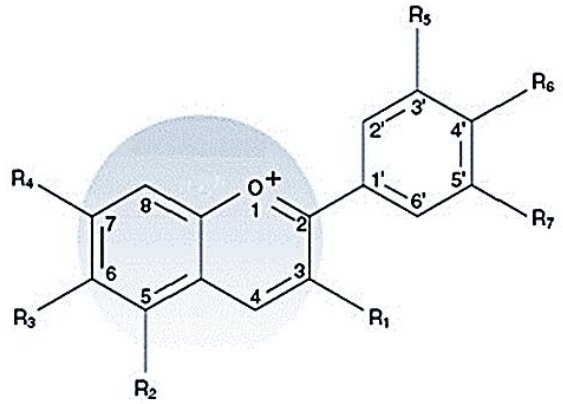
O interesse recente no estudo de antocianinas deve-se ao potencial de aplicação que estes compostos podem ter como corantes na indústria de alimentos. Além disso, as antocianinas fazem parte da dieta humana há milhares de anos e é

possível afirmar que os seres humanos estão bem adaptados ao consumo destes compostos. Alguns extratos vegetais que contém antocianinas já são utilizados comercialmente, como os obtidos do bagaço de uvas (subprodutos da indústria viticultora), repolho roxo, batata-doce e hibisco, entre outros.

As antocianinas são moléculas presentes nos grupos dos flavonóis (polifenóis) e compreendem o maior conjunto de pigmentos solúveis em água do reino vegetal. Diferentemente das clorofilas e dos caratenóides, a absorção eletrônica das antocianinas esta distribuída por toda a região visível do espectro eletrônico (400 nm – 700 nm). Além de serem responsáveis pelas cores vermelha, azul e roxa da maioria das flores e frutas (Timberlake e Bridle, 1976). As moléculas de antocianinas são encontradas nas plantas no interior dos vacúolos de células epidérmicas, tanto na forma de cristais como dissolvidas, dando origem a íons com carga positiva, o cátion flavílio, que podem desprotonar e formar uma estrutura eletricamente neutra, denominada base quinoidal (Andersen e Markham, 2006).

A cor exibida por estas moléculas foi explicada por Pauling, em 1939, que propôs que a estrutura de ressonância do íon flavílio causou a intensidade da sua cor (Wrolstad, Durst *et al.*, 2005).

Há uma enorme variedade de antocianinas, distribuídas na natureza (nomes e as abreviaturas dos mais comuns estão listados na Tabela 1). As principais diferenças entre elas são o número de grupos hidroxilados, a natureza e o número de açúcares ligadas à sua estrutura, os carboxilatos alifáticos ou aromáticos ligados ao açúcar na molécula e a posição destas ligações (Kong, Chia *et al.*, 2003). Até o momento, há relatos de mais de 500 antocianinas diferentes (Andersen e Markham, 2006) e 23 antocianidinas (Giusti, Rodriguez-Saona *et al.*, 1999; Wrolstad, Durst *et al.*, 2005; Andersen e Markham, 2006), das quais apenas seis são os mais comuns em plantas, Pelargonidina (Pg), Petunidina (Pt), Cianidina (Cy), Malvidina (Mv), Peonidina (Pn) e Delfinidina (Dp). Os derivados de glicosídeos das três antocianinas não-metiladas (Cy, Dp e Pg) são as mais comuns na natureza, sendo encontrado em 80% de folhas pigmentadas, e 69% em frutas e 50% em flores (Dey e Harborne, 1993).



Nome	Abreviação	Padrão de substituição							Cores
		R1	R2	R3	R4	R5	R6	R7	
Apigeninidina	Ap	H	OH	H	OH	H	OH	H	NR[a]
Arrabidina	Ab	H	H	OH	OH	H	OH	OMe	
Aurantinidina	Au	OH	OH	OH	OH	H	OH	H	
Capensinidina	Cp	OH	OMe	H	OMe	OH	OH	OMe	azul-vermelho
Carajurina	Cj	H	H	OH	OH	H	OMe	OMe	NR[a]
Cianidina	Cy	OH	OH	H	OH	OH	OH	H	laranja-vermelho
Delfinidina	Dp	OH	OH	H	OH	OH	OH	OH	azul-vermelho
Europinidina	Eu	OH	OMe	H	OH	OMe	OH	OH	azul-vermelho
Hirsutidina	Hs	OH	OH	H	OMe	OMe	OH	OMe	azul-vermelho
3_0-HydroxyAb	3'OHAb	H	H	OH	OH	OH	OH	OMe	NR[a]
6-Hydroxy Cy	6OHCy	OH	OH	OH	OH	OH	OH	OH	vermelho
6-Hydroxy Dp	6OHDp	OH	OH	OH	OH	OH	OH	OH	azul-vermelho
6-Hydroxy Pg	6OHPg	OH	OH	OH	OH	H	OH	H	NR[a]
Luteolina	Lt	H	OH	H	OH	OH	OH	H	
Malvidina	Mv	OH	OH	H	OH	OMe	OH	OMe	azul-vermelho
5-Metil-Cy	5-MCy	OH	OMe	H	OH	OH	OH	H	laranja-vermelho
Pelargonidina	Pg	OH	OH	H	OH	H	OH	H	
Peonidina	Pn	OH	OH	H	OH	OMe	OH	H	laranja-vermelho
Petunidina	Pt	OH	OH	H	OH	OMe	OH	OH	azul-vermelho
Pulchellidina	Pl	OH	OMe	H	OH	OH	OH	OH	azul-vermelho
Ricionidina A	RiA	OH	H	OH	OH	H	OH	H	NR[a]
Rosinidina Rs	Rs	OH	OH	H	OMe	OMe	OH	H	vermelho
Tricetinidina	Tr	H	OH	H	OH	OH	OH	OH	vermelho

Tabela 1. Identificação estrutural de antocianidinas (agliconas). (Castañeda-Ovando, Pacheco-Hernández *et al.*, 2009). *NR: Não reportado

Além disso, as antocianinas isoladas são altamente instáveis e muito susceptíveis a degradação (Giusti, Rodriguez-Saona *et al.*, 1999; Giusti e Wrolstad, 2001). A sua estabilidade é afetada por vários fatores tais como pH, temperatura de armazenagem, a estrutura química, a concentração, a luz, o oxigénio, os solventes, a presença de enzimas, flavonóides, proteínas e ions metálicos (Rein, 2005).

A enorme variedade de antocianinas encontrados na natureza faz delas um grupo muito complexo e interessante. Recentemente, estudos têm indicado que a cianidina-3-o-glicosideo (Cyn-3G), abundante em legumes coloridos é um composto fotoquímico que é capaz de reduzir danos oxidativos gerados pelo envelhecimento e isquêmica cerebral (Garcia-Beneytez, Cabello *et al.*, 2003)

E em diversos estudos tem sido demonstrado que as antocianinas possuem potente capacidade antioxidante e propriedades anti-inflamatórias (Harborne e Williams, 2000), e muitos frutos e vegetais altamente pigmentados têm sido identificados por apresentarem elevada atividade antioxidante, através do ensaio de capacidade de absorção do radical oxigénio (ORAC) (Wu e Prior, 2005). Há uma quantidade considerável de estudos na literatura com compostos que apresentam propriedades antioxidantes e atuam como agentes anti-inflamatórios na dieta, e têm sido utilizados para reverter déficits comportamentais e neuronais associados com o envelhecimento. Mais importante, estudos prévios (Youdim, Dobbie *et al.*, 2003), tem indicado que a suplementação dietética de curto prazo com extrato de mirtilo foi eficaz de reverter os déficits relacionados a idade, funções neuronais e comportamentais (tais como cognitivo e motor). A suplementação com extrato de mirtilo foi também relacionado com melhorias significativas em vários índices de sinalização neuronal (*e.g*. sensibilidade do receptor muscarínico, e ativação de quinases reguladas por sinal extracelular, ERK) (Joseph, Shukitt-Hale *et al.*, 2005). Entre os muitos compostos encontrados em mirtilos que podem ser mediadores de efeitos benéficos estão as catequinas, proantocianidinas e antocianinas. Tem sido demonstrado, que as antocianinas, dentre outros compostos polifenólicos apresentam uma série de efeitos periféricos (Youdim, Dobbie *et al.*, 2003), incluindo: (a) estabilização do tecido conjuntivo, (b) promover a formação de colágeno e (c) na prevenção ao dano oxidativo em vasos sanguíneos.

Além disso, alguns autores sugerem que o uso da casca escura do grão de soja (rico em antocianinas: cianidina-3-glicosídeo, delfinidina-3-glicosídeo e petunidina-3-glicosídeo) pode servir de estratégia farmacológica para modular

desordens cardiovasculares (Rechner, Smith *et al.*, 2004). Nestes trabalhos foi verificado que as antocianinas e metabólitos colônicos de polifenóis *in vivo* apresentam propriedades anti-trombóticas, por inibir a agregação plaquetária. Entre esses trabalhos, foi observaram que a ativação das plaquetas (expressão da P-seletina) estava significativamente reduzida de 10 a 40% das plaquetas em repouso, e plaquetas advindas do estresse provocado por peróxido de hidrogênio e por plaquetas pré-ativadas pela epinefrina, relativo aos controles. Nesse estudo, os autores concluem que as antocianinas e metabólitos de polifenóis, bem como fontes da dieta e seus precursores são promotores em potencial para a saúde cardiovascular (Rechner, Smith *et al.*, 2004).

Adicionalmente, Kim et al. (2006) examinaram a inibição da expressão de alguns genes inflamatórios provocada pela injúria da isquemia/reperfusão (I/R). Antocianinas isoladas da casca escura do grão de soja diminuíram níveis vasculares de moléculas de adesão celular-1 (VCAM-1), moléculas de adesão intracelular-1 (ICAM-1) e níveis de ciclooxigenases-2, induzidas pelo fator de necrose tumor alfa (TNF-alfa), aos quais são dependentes da via NF-kappaB (Kim, Son *et al.*, 2004).

As antocianinas dos alimentos são biodisponíveis, principalmente em sua forma intacta glicosilada. Estas podem ser absorvidas a partir do estômago (Passamonti, Vrhovsek *et al.*, 2003), estando presente no plasma (Matsumoto, Inaba *et al.*, 2001), no fígado e no rim (Tsuda, Horio *et al.*, 1999) e também na urina excretada (Felgines, Texier *et al.*, 2002). Kalt, Blumberg *et al.*, (2008) demonstram que o modelo mais adequado para avaliar a absorção digestiva humana é utilizando porcos como cobaias, e este modelo foi usado para examinar a deposição de antocianinas em tecidos, incluindo o fígado, olho e tecido cerebral. Os porcos foram alimentados com dietas suplementadas com 0, 1, 2, ou 4% de mirtilos (*Vaccinium corymbosum L*. 'Jersey') por 4 semanas. Apesar das antocianinas não serem detectados no plasma ou urina dos animais em jejum, os resultados de LC-MS/MS mostraram que uma concentração relativa de 11 antocianinas intactas foram encontradas no fígado, olhos, córtex cerebral e cerebelo. Estes resultados sugerem que as antocianinas podem acumular-se em tecidos, incluindo tecidos superiores à barreira hemato-encefálica (Kalt, Blumberg *et al.*, 2008).

Como vimos, o estresse oxidativo é amplamente aceito como um dos responsáveis pela sinalização e ativação de vias deletérias e prejuízos comportamentais observadas na senescência. Por conseguinte, inúmeros estudos

tem investigado exaustivamente a eficácia dos antioxidantes no que diz respeito à redução dos efeitos deletérios do envelhecimento cerebral. Esta tese, e estudos de outros autores, sugerem que as combinações de antioxidantes e componentes anti-inflamatórios encontrados em frutas e vegetais podem diminuir ou reverter déficits relacionados à função cerebral e comportamento cognitivo (Joseph, Shukitt-Hale *et al.*, 2005; Gutierres, J. M., Carvalho, F. B. *et al.*, 2012)

O envelhecimento está associado com muitas doenças crônicas degenerativas comuns e, as causas precisas da degeneração neuronal subjacente a estas doenças ainda não está completamente clara. Pensa-se que muitos eventos celulares e moleculares envolvidos, incluem o aumento do estresse oxidativo, diminuição das funções mitocôndrias, ativação de apoptose neuronal, deposição de proteínas agregadas e excitotoxicidade. Até agora, a maioria dos tratamentos com drogas existentes para o tratamento de perturbações neurodegenerativas são incapazes de prevenir a degeneração de neurónios e, consequentemente, existe um desejo de desenvolver terapias alternativas capazes de impedir a perda progressiva de populações neuronais específicas. Uma vez que a neuropatologia de muitas doenças do cérebro tem sido associada ao aumento do estresse oxidativo cerebral e, historicamente, grandes esforços tem sido dirigidos a explorar estratégias alternativas como o uso de compostos antioxidantes para combater estes danos neuronais (Swerdlow, 2011).

De fato, tem havido um crescente interesse nos efeitos neuroprotetores de plantas que apresentam um grupo de metabólitos secundários conhecidos como polifenóis, que são antioxidantes poderosos *in vitro*. Um grande número de estudos de intervenção dietética em seres humanos (Macready, Kennedy *et al.*, 2009) e em animais (Maher, 2009), em particular aqueles que utilizam os alimentos e bebidas derivadas de *Vitis vinifera* (uva), *Camellia sinensis* (chá), *Theobroma cacao* (cacau) e *Vaccinium spp.* (Blueberry), demonstraram efeitos benéficos sobre a função vascular humana e na melhoria da memória e do aprendizado (Maher, 2009; Rendeiro, Spencer *et al.*, 2009; Spencer, 2009; Rendeiro, Vauzour *et al.*, 2012).

Evidências começaram a emergir indicando que o baixo peso molecular, e os componentes não-nutricionais podem ser responsáveis pelos efeitos benéficos dos alimentos ricos em flavonóides, *in vivo*, através da sua capacidade de interagir, direta ou indiretamente, com a arquitetura inata do cérebro para a memória (Zini, Del Rio *et al.*, 2006). As ações biológicas de flavonóides no cérebro foram atribuídos à

sua capacidade de exercer seu potencial antioxidante (Shimmyo, Kihara *et al.*, 2008) através da sua habilidade de eliminar espécies reativas ou através de influências no estado redox intracelular (Williams e Spencer, 2012). No entanto, estudos mais recentes demonstram que a atividade antioxidante clássica de doar hidrogénio a uma espécie reativa, não pode explicar a bioatividade de flavonóides *in vivo*, particularmente no cérebro, onde são encontrados em concentrações muito baixas (Maher, Akaishi *et al.*, 2006). Em vez disso, tem sido postulado que os seus efeitos no cérebro são mediados por uma capacidade de proteger a vulnerabilidade de neurônios, melhorando a função neuronal existente, e estimulando o fluxo sanguíneo cerebral e induzindo neurogênese (Williams e Spencer, 2012) (ver Figura 3).

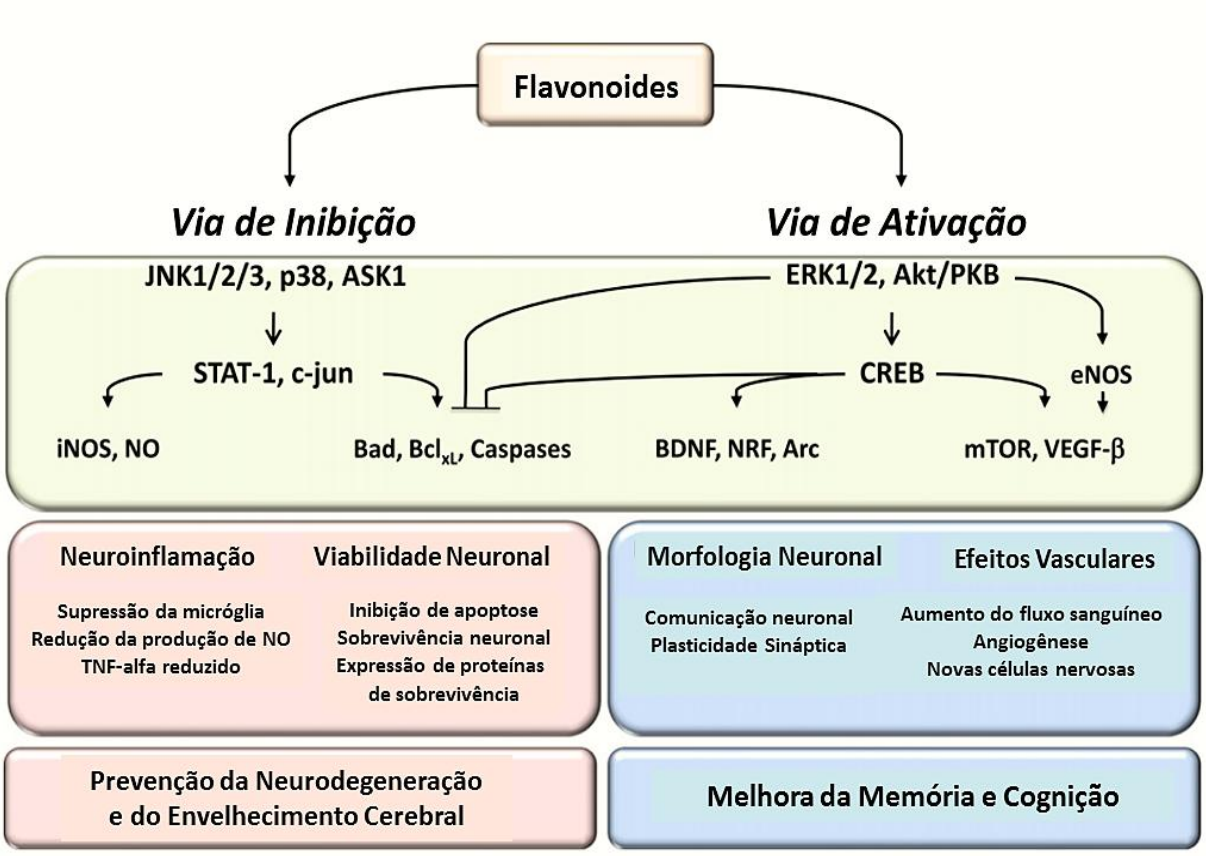


Figura 3. Flavonoides induzem em células neuronais e gliais a ativação e inibição de vias de sinalização e de funcionalidade. Adaptado de (Williams e Spencer, 2012).

Estudos *in vitro,* mostram que os flavonóides e os seus metabolitos fisiológicos são capazes de ativar importantes vias de sinalização neuronais e gliais na indução de plasticidade sináptica, porém em baixas concentrações nanomolares,

semelhante ao relatado para o cérebro. No entanto, a sua interação com estas vias tem maior importância, uma vez que estas são também as vias de sinalização responsáveis pela determinação do destino que os neurônios irão seguir uma vez expostos a neurotoxinas e também em relação aos mediadores inflamatórios e ao controle do fluxo de sanguíneo cerebral, importantes para o correto funcionamento cerebral que é afetado principalmente na Doença de Alzheimer e demências associadas ao envelhecimento do sistema nervoso central (Williams e Spencer, 2012).

A maioria das histórias de demência senil começa com a descrição de Alois Alzheimer em 1906 sobre o primeiro caso da doença de Alzheimer, mas a história de demência senil antes de 1906 é bastante rica, e remonta aos antigos médicos e filósofos gregos e romanos (Berchtold e Cotman, 1998). Ao longo dos 2500 anos desde os tempos antigos, o conceito de demência senil evoluiu a partir de uma noção bastante vaga de que o declínio cognitivo ocorre inevitavelmente na velhice, e nos dias atuais esse conceito é definido por um conjunto distinto de características clínicas e patológicas. Ao longo da história, muitos idosos com comportamento imprevisível foram deixados em instituições asilares, e a linha entre transtornos mentais e demência senil sempre foi nebulosa. A identificação da doença de Alzheimer no início do século 20 foi decisiva para a compreensão da demência senil, e os conceitos e achados histológicos apresentados pelos pesquisadores iniciais da doença de Alzheimer (DA) continuam a ser relevantes ainda hoje (ver Figura 4). Na verdade, estes resultados iniciais estão provando ser uma fonte contínua de "*insight*", como muitos dos assuntos debatidos na virada do século que ainda continuam por serem esclarecidos (Berchtold e Cotman, 1998).

Uma das modificações presentes na maioria das doenças do cérebro é a deteriorização cognitiva. Esta deteriorização cognitiva é a causa mais comum de mortalidade durante a velhice, contribuindo para um desfecho dramático, por acarretarem perda de autonomia do doente e custos elevados de acompanhamento diário e internamento.

Um relatório divulgado pela Organização Mundial da Saúde (OMS) e a Associação Internacional da doença de Alzheimer (ADI) consideram a DA como uma prioridade mundial no âmbito de saúde pública. A OMS reconhece o tamanho e a complexidade do desafio da demência e exorta os países em vê-la como uma prioridade crítica de saúde pública, uma vez que um caso de DA surge a cada

quatro segundos no mundo (Wortmann, 2012). Segundo a ADI aproximadamente 35,6 milhões de pessoas em 2010 convivem com o Alzheimer, e a estimativa é de que este número praticamente dobre a cada 20 anos, chegando a 65,7 milhões em 2030 e a 115,4 milhões em 2050 (Wortmann, 2012).

A DA está relacionada com a idade e é caracterizada por uma gama de alterações na anatomia do cérebro, biologia e função. Nenhum tratamento ou opções de intervenção, que possam atuar nos eventos moleculares centrais que constituem a sua fisiopatologia estão disponíveis atualmente. A mais proeminente caracteristica neuropatologica da DA é a presença de placas senis compostas por peptideos β-amilóide e emaranhados neurofibrilares (Swerdlow, 2007) derivado da agregação de microtubulos associados à proteina tau, como representado na Figura 4.

Estudos epidemiológicos tem demonstrado que a Diabetes mellitus tipo 2 (DMT2), uma sindrome metabólica associada ao envelhecimento caracterizada por resistência periférica à insulina, é um fator de risco importante para o desenvolvimento de danos cognitivos e demência, incluindo a DA (Liu, Liu *et al.*, 2011). A cascata de neurodegeneração da DA está associada com persistente estresse oxidativo, disfunção mitocondrial, comprometimento no metabolismo energético, e a ativação de genes de vias de sinalização pró-morte. Mais recentemente, estudos com o tecido cerebral de humanos *postmortem* associou como característica molecular e patológica na DA à redução da expressão de insulina e genes do IGF (*insulin-like growth factor*) e seus receptores correspondentes (Liu, Liu *et al.*, 2011).

É amplamente reconhecido que o envelhecimento resulta em déficits no funcionamento da memória em uma variedade de tarefas cognitivas (Salthouse, 2003). No entanto, a natureza dos mecanismos que conduzem a estes déficits relacionadas com a idade permanece controversa.

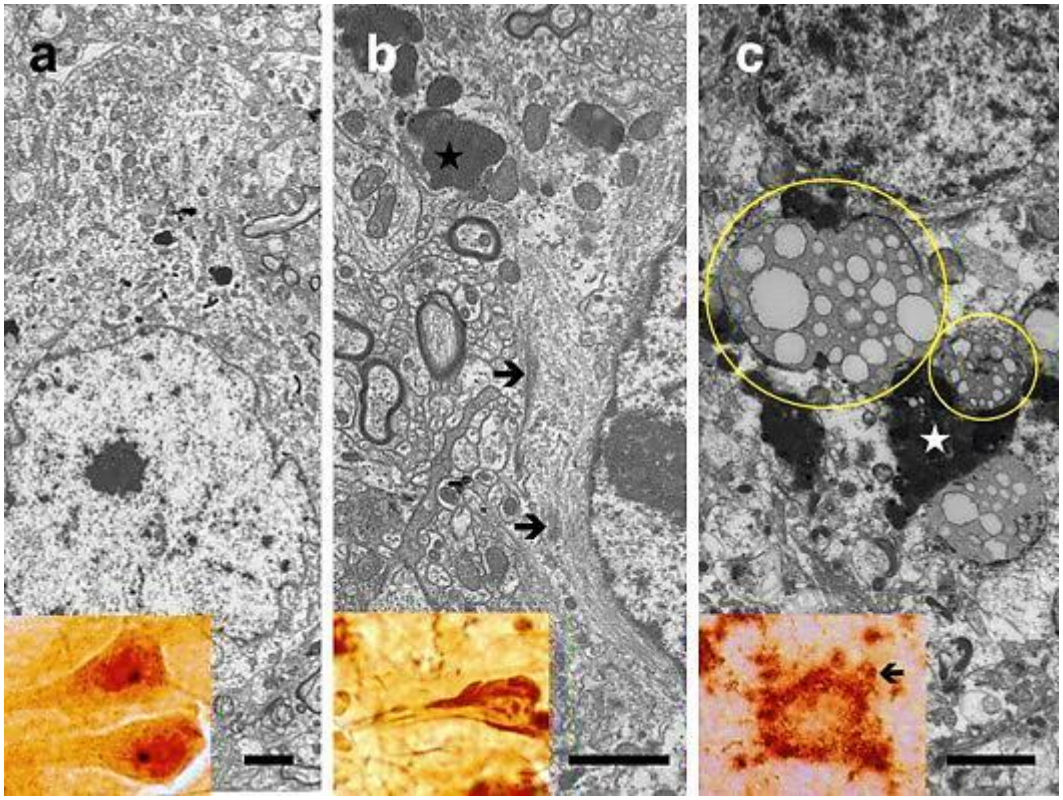


Figura 4. Fotografias de microscopia eletrônica de neurônios de sujeitos normais e com doença de Alzheimer (DA). (a) neurônios normais de sujeito controle; (b) Degeneração neural contendo emaranhados neurofibrilares (setas) em pacientes com DA; (c) Degeneração neuronal mostrando a deposição de peptídeo β-amilóide e acumulação de vacúolos autofágicos na DA. Retirado do artigo de revisão (Yamashima, 2013).

Evidências indicam que o envelhecimento pode afetar diferencialmente o processamento da memória como a codificação da elaboração e da recuperação estratégica da memória durante o seu processamento (Salthouse, 2003). Alternativamente, é possível que o declínio cognitivo global, seja o resultado do processo de envelhecimento associado a doenças neurodegenerativas (Krueger e Salthouse, 2011), corroborando para o declínio na velocidade de processamento de informações e também em demência (Soubelet e Salthouse, 2011).

A demência é uma desordem cerebral caracterizada por um declínio em várias funções mentais superiores (por exemplo, memória, inteligência e personalidade) causando prejuízos significativos no funcionamento diário cerebral (Canadian study of health and aging: study methods and prevalence of dementia, 1994). A prevalência da demência aumenta com a idade, dobrando a cada cinco anos entre as idades de 60 e 90 anos (Corrada, Brookmeyer *et al.*, 2008). Com base

nos dados epidemiológicos, a demência é amplamente reconhecida como um grande problema social, médico e económica nos países desenvolvidos (Gao, Hendrie *et al.*, 1998). Infelizmente, a demência está se tornando um grande problema nos países em desenvolvimento, onde ela não existia há 50 anos (Zilkens, Davis *et al.*, 2013).

Mais de 50 milhões de pessoas no mundo têm demência, e a causa mais comum e irreversível desta demência é a DA (Adlard, James *et al.*, 2009). A DA é dividida em duas formas principais, nomeadamente familiares (DAF) e esporádicas (DAS) caracterizadas por déficits cognitivos e perda neuronal extensa no SNC (Michon, 2009; Reed, Pierce *et al.*, 2009) e ao nível molecular pela presença de anormalidades específicas do citoesqueleto, incluindo emaranhados neurofibrilares intracelulares (NFT), formadas por proteína tau hiperfosforilada e a presença de níveis elevados de fragmentos de aminoácidos Aβ40-42 (Woodhouse, Shepherd *et al.*, 2009).

A forma de início precoce (isto é, DAF) tem uma forte correlação existente entre a genética e traços característicos da patogênese DA bem como mutações na proteína precursora de amilóide (APP) (Bernardi, Geracitano *et al.*, 2009), presenilina (PS-1), e PS-2 (Huang e Jiang, 2009) De particular interesse, a outra forma de DA, a DAS é uma doença multifatorial, em que fatores genéticos e epigenéticos cestão envolvidos (Zawia, Lahiri *et al.*, 2009). As mutações genéticas que ocorrem na DAS são mutações no gene da apolipoproteína E (APOE) mais especificamente no alelo 4 (Wharton, O'callaghan *et al.*, 2009) e no gene PS-2 promotor de polimorfismo (Liu, Liu *et al.*, 2008). Muitos estudos indicam que os distúrbios de vários aspectos do metabolismo celular parecem ter certa importância patológica na DAS.

Entre estes, a resistência à insulina aumentada no cérebro (Salkovic-Petrisic, 2008), diminuição do metabolismo energético e utilização da glicose são observadas nos estádios iniciais da doença (De La Torre, 2008). Em consequência do déficit de energia, o estresse oxidativo (Droge e Kinscherf, 2008) e inflamação (De La Monte, Neusner *et al.*, 2009) são mais pronunciados no tecido neuronal podendo causar neurodegeneração em DAS.

Alguns dos aspectos patológicos da DAS em seres humanos podem ser induzidas pela administração intracerebrovetricular (ICV) de estreptozotocina (STZ) em ratos, que é comumente empregado para estudar a demência experimental.

Mais importante ainda, é que doses subdiabetogenicas de ICV-STZ podem induzir alterações no receptor cerebral de insulina (RI), mudanças na sinalização da insulina e, conseqüentemente, alterações comportamentais, neuroquímicas, alterações bioquímicas, morfológicas e histológicas semelhantes ao envelhecimento cerebral (Salkovic-Petrisic, 2008).

Além disso, tem sido demonstrado que o modelo de ICV-STZ em ratos tem como alvo o funcionamento do cérebro e a sinalização em cascata induzida pelo RI. No cérebro, a diminuição dos níveis de glicose/metabolismo energético, particularmente nas regiões do córtex cerebral e hipocampo têm sido relatados a partir de 1 semana após a administração ICV-STZ (Pathan, Viswanad *et al.*, 2006) e, consequentemente, gera disfunção mitocondrial (Agrawal, Tyagi *et al.*, 2009). Além disso, um progressivo estresse oxidativo tem sido encontrado tanto em horas como uma semana após a administração ICV-STZ (Pathan, Viswanad *et al.*, 2006; Gutierres, J. M., Carvalho, F. B. *et al.*, 2012). Assim, além do metabolismo energético reduzido e disfunção mitocondrial, o aumento da produção de radicais livres, estresse oxidativo e nitrosativo são considerados fatores que poderiam prejudicar a aprendizagem e a memória conduzindo a disfunção cognitiva (Ishrat et al, 2009 a, b,. Tiwari et al, 2009).

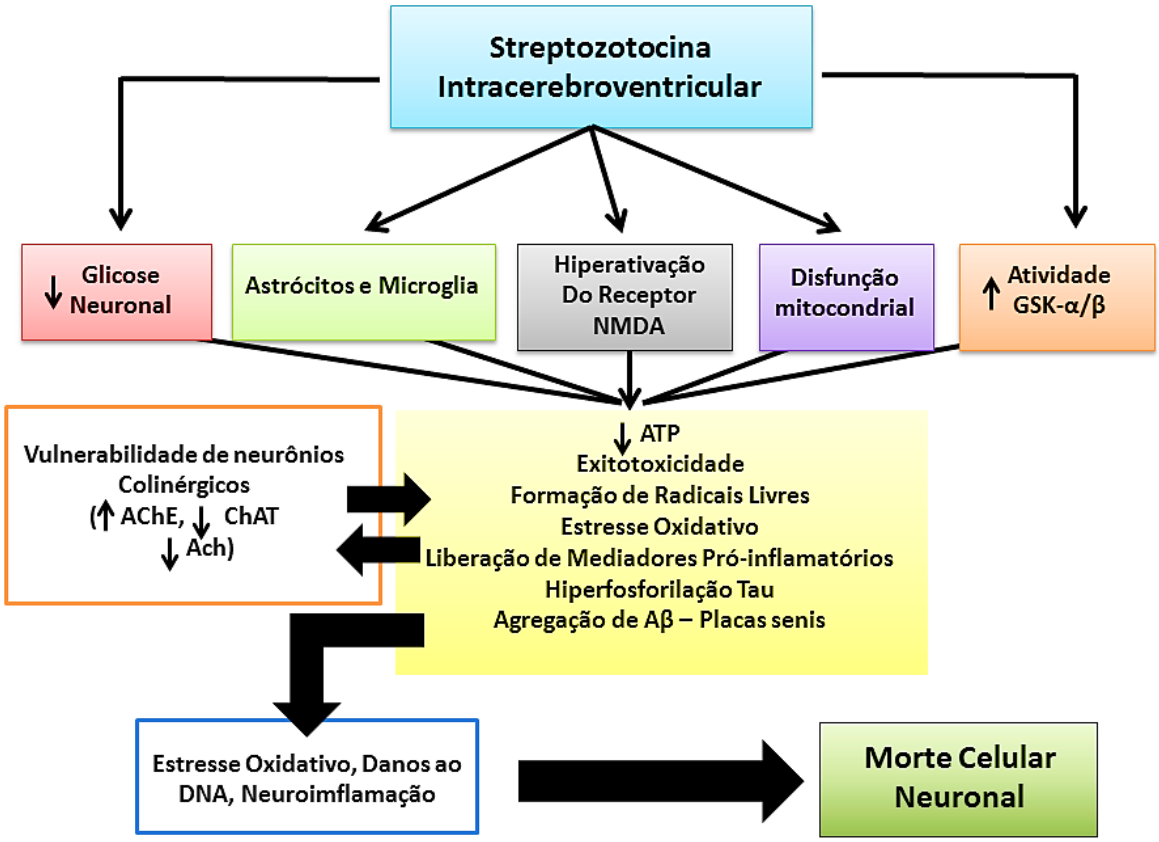


Figura. 5. Mecanismo central de ação da estreptozotocina intracerebroventricular administrado em ratos (Mehan, Arora *et al.*, 2012).

Uma diminuição na transmissão colinérgica (colina-acetiltransferase diminuída e aumento da atividade da acetilcolinesterase) começou a ser encontrada mais tarde no hipocampo de ratos ICV-STZ tratados (Mehan, Arora *et al.*, 2012). A administração de ICV-STZ é também associada com alterações morfológicas cerebrais, seguido por perda de células extensiva e neurodegeneração por indução de danos específicos na mielina e astrogliose reativa encontrada uma semana após o tratamento (Sonkusare, Srinivasan *et al.*, 2005). A administração de ICV-STZ ao reduzir a disponibilidade de energia pode também causar um aumento no cálcio citoplasmático (Ca^{2+}) (Muller, Nitsch *et al.*, 1998), confirmados por uso farmacológico de bloqueadores do canal de cálcio (lercanidipina) que marcadamente atenuam alterações comportamentais e bioquímicas em ratos tratados dom ICV-STZ (Sonkusare, Srinivasan *et al.*, 2005). É bem sabido que as funções cerebrais dependentes de ATP são marcadamente afetadas por falhas no estado energético cerebral bem como o metabolismo de glicose reduzido. Todas essas alterações

neuroquímicas e estruturais foram observadas logo após duas semanas de administração ICV-STZ e estudos tem mostrado que essas alterações persistem por mais 12 semanas de tratamento, acompanhadas por longo prazo de déficits progressivos na aprendizagem e memória (Hoyer, Lannert *et al.*, 1999) e desempenham assim um papel importante na patogênese da DAS.

Diversos estudos vêm investigando o papel do sistema colinérgico sobre a modulação de funções cognitivas, através do uso de antagonistas de receptores muscarínicos, de agonistas nicotínicos, ou da enervação colinérgica, em estruturas envolvidas na consolidação de memórias e na execução de atenção e vigília (Gold, 2003).

A síntese de ACh se dá a partir da transferência de um grupo acetil de uma molécula de acetil coenzima A (acetil CoA), proveniente da metabolização celular da glicose, para uma molécula de colina. Esta reação é catalisada pela enzima colina acetiltransferase (ChAT), presente principalmente no citoplasma da célula colinérgica (**figura 6**). A ACh recém sintetizada é então armazenada em vesículas, através da atividade do transportador vesicular de acetilcolina (VAChT), que utiliza o gradiente eletroquímico da bomba H^+-ATPase e realiza a troca de dois prótons H^+ presentes no lúmen vesicular por uma molécula de ACh do citoplasma (Nguyen, Cox *et al.*, 1998). Na fenda sináptica, a acetilcolinesterase (AChE) hidrolisa a ACh e a colina liberada é então reciclada após captação pelo transportador de colina de alta afinidade (ChT1) do neurônio colinérgico (Rand, 2007).

Muitas evidências têm emergido e destacado a importância da participação do sistema colinérgico em processos cognitivos. A acetilcolina (ACh) participa do processamento de memórias procedurais no estriato, conforme observado no teste de “rotarod” (cilindro giratório) (Carta, Stancampiano *et al.*, 2006). Estudos apontam o papel da ACh sobre a formação de memórias explícitas ou declarativas. A injeção de escopolamina, um antagonista de receptores muscarínicos, compromete a memória episódica para o reconhecimento de objetos (Ennaceur e Meliani, 1992; De Bruin e Pouzet, 2006), enquanto a administração de agonistas nicotínicos, por sua vez, é capaz de potencializar essa memória (Puma, Deschaux *et al.*, 1999). A escopolamina impede também a capacidade de ovelhas reconhecerem seus filhotes, quando injetada em um período de até 8 horas pós-parto (Ferreira, Gervais *et al.*, 1999). O sistema colinérgico parece participar da modulação do comportamento agressivo, através de sua atuação no núcleo da estria terminal, aumentando a

latência para a exibição de comportamento defensivo após estimulação elétrica do hipotálamo de gatos (Kono, Tashiro *et al.*, 1986).

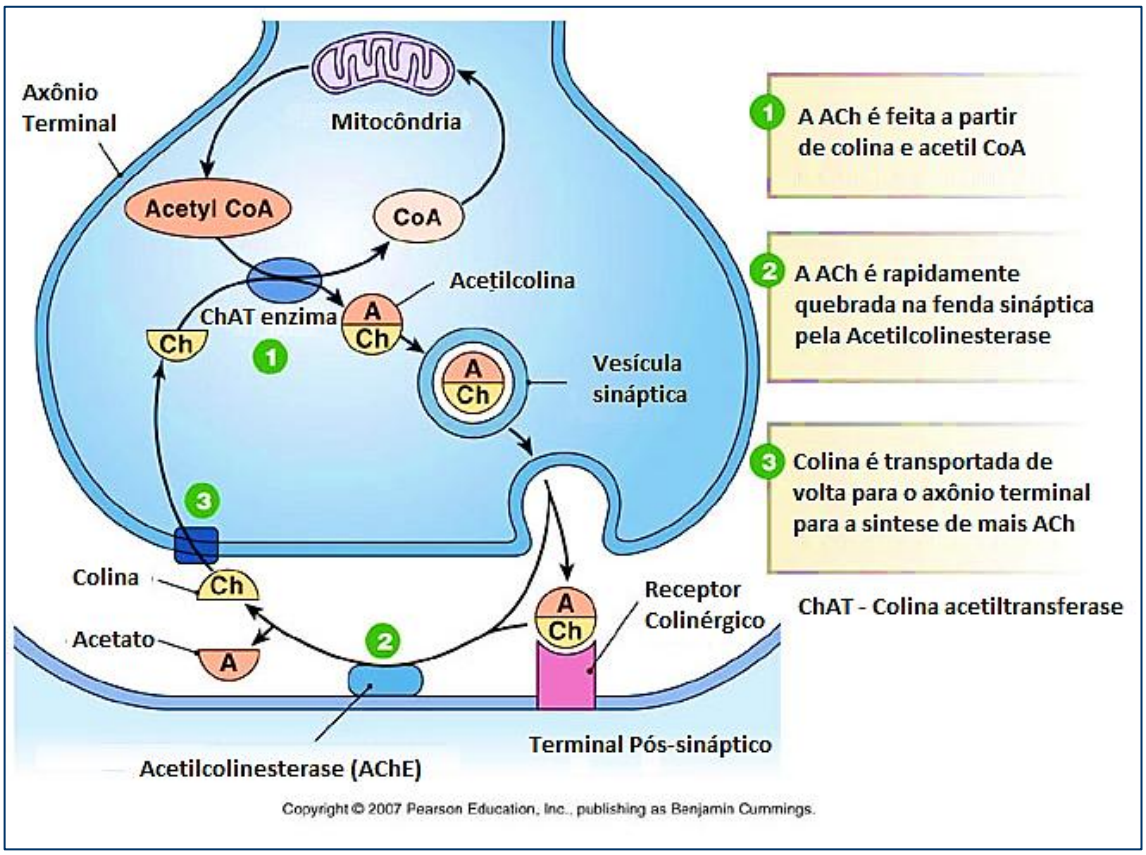


Figura 6. Representação da Neurotransmissão colinérgica adaptado de (Jones, Byun *et al.*, 2012).

Além disso, no neurônio pós-sináptico da neurotransmissão colinérgica, existem dois tipos de receptores, os nicotínicos (nAChRs) e os muscarínicos (mAChRs). Os nAChRs são receptores do tipo canal iônico que permitem a despolarização da membrana neuronal devido à sua permeabilidade a Na^+, K^+ e Ca^{2+}. Os mAChRs são receptores metabotrópicos acoplados à proteína G e podem ser do tipo excitatório ou inibitório (**Figura 7**) (Rand, 2007)

As proteínas ChAT, ChT1 e VAChT são específicas de neurônios colinérgicos. Logo, estas proteínas tem sido utilizadas como marcadores de neurônios colinérgicos, permitindo a identificação das regiões de síntese de ACh, bem como os alvos centrais de suas inervações (Schafer, Eiden *et al.*, 1998).

As projeções colinérgicas para o córtex perirrinal participam do processamento da memória para o reconhecimento de objetos em ratos (Winters e Bussey, 2005) e também da memória visual em macacos (Tang, Mishkin *et al.*, 1997). No hipocampo, a ACh é importante para a formação da memória espacial (Hunsaker, Rogers *et al.*, 2007) e do condicionamento contextual ao medo (Rogers e Kesner, 2004).

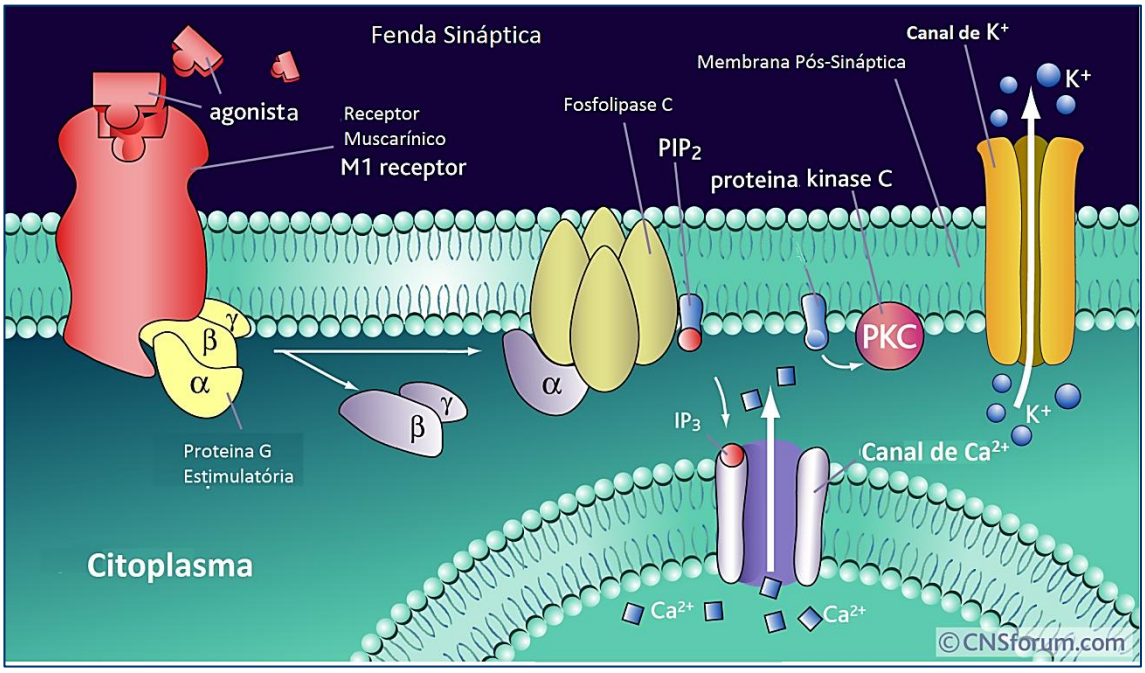


Figura 7. Representação do receptor muscarínico metabotrópico (receptor M1), acoplado a uma proteína G, e as vias de sinalização induzidas pela ativação do receptor por seu agonista especifico (Rang, Dale *et al.*, 2001).

A ACh modula também a capacidade de atenção e vigília, através de inervações colinérgicas do córtex pré-frontal, parietal e somatosensorial (Klinkenberg, Sambeth *et al.*, 2011). No bulbo olfatório, o processamento de pistas olfatórias é modulado pelas projeções colinérgicas, que parecem potencializar a discriminação entre odores quimicamente similares (Mandairon, Ferretti *et al.*, 2006).

As estratégias terapêuticas para combater os transtornos cognitivos têm sido desenvolvidos com a perspectiva de melhorar a disponibilidade de ACh no SNC. Os agonistas de receptores colinérgicos (muscarínicos e nicotínicos), potencializam o nível endógeno de ACh (promotores e inibidores da síntese de enzimas

metabolizadoras) e têm sido utilizados para tratar a demência esporádica do tipo Alzheimer. Dentre as várias abordagens testadas, a inibição da AChE (a enzima de metabolização de ACh) é a única abordagem considerada eficaz (Giacobini, 1996). Uma vez que os inibidores da acetilcolinesterase são utilizados como base do tratamento para a DA, os mesmos possuem um importante papel de interação com os processos neuropatológicos em curso, podendo ser mais vantajoso para a neuroproteção.

A sinalização purinérgica também constitui atualmente como um importante alvo de estudos que apresentam interesse em investigar o papel deste sistema em modular uma variedade de processos biológicos, incluindo a tromboregulação, inflamação e neurotransmissão (Burnstock, 2006; Schetinger, Morsch *et al.*, 2007)

O sistema purinérgico caracteriza-se pelo envolvimento de três componentes principais: os nucleotídeos e nucleotídeos extracelulares, os receptores através dos quais estes nucleotídeos exercem seus efeitos e as ecto-enzimas responsáveis pelo controle dos níveis extracelulares destas moléculas (Burnstock, 2006).

Em 1954 os pesquisadores Holton & Holton demonstraram, pela primeira vez, o ATP como um neurotransmissor, mostrando a sua liberação a partir de nervos sensoriais. Anos mais tarde em 1972, Burnstock propôs pela primeira vez que, além da transmissão colinérgica e noradrenérgica, existe no sistema nervoso autônomo a transmissão purinérgica, onde o ATP é o principal neurotransmissor. Além disso, o ATP é co-liberado com diversos neurotransmissores em neurônios do SNC e periférico (Abbracchio e Burnstock, 1998). O mecanismo exato de liberação do ATP permanece ainda uma incógnita, apesar de inúmeros estudos tentando esclarecer o seu mecanismo (Bodin e Burnstock, 2001).

Adicionalmente, há vários argumentos provando que o ATP armazenado nas vesículas sinápticas é liberado como um co-transmissor de terminais nervosos por exocitose, em conjunto com outros neurotransmissores, como a acetilcolina e noradrenalina (Zimmermann, 1994).

Figura 8. Receptores isolados e estaticamente espalhados na membrana extracelular (painel superior, adaptado de *H. Matisse, La Musique, 1910, tela a óleo*), em seguida, se agrupam em um "bloco receptor", depois da ativação pelo seu ligante específico o ATP, e a transmissão da sensação de liberação funcional em um sistema (painel inferior, adaptado de *H. Matisse, La Musique, 1910, tela a óleo*) (Volonte, Amadio *et al.*, 2008)

Quando liberados, os nucleotídeos de adenina interagem com receptores específicos e são hidrolisados por ecto-enzimas até seus respectivos nucleotídeos (Grobben, Anciaux *et al.*, 1999). Os nucleotídeos extracelulares de adenina ATP e ADP, e nucleotídeo correspondente à adenosina são considerados importantes moléculas sinalizadoras, para uma variedade de processos biológicos, incluindo a neurotransmissão, a contração muscular, modulação da função cardíaca e plaquetária, vasodilatação, no metabolismo do glicogênio hepático, dor, proliferação, diferenciação, apoptose entre outros (Agteresch, Dagnelie *et al.*, 1999).

Os nucleotídeos e nucleotídeos de adenina podem exercer seus efeitos através da ativação de receptores purinérgicos subdivididos em dois grandes grupos: P1 e P2. Os purinoreceptores do tipo 1 são mais eficientemente ativados por

adenosina, enquanto os receptores P2, principalmente por ATP (Figura 9) (Burnstock e Kennedy, 1985).

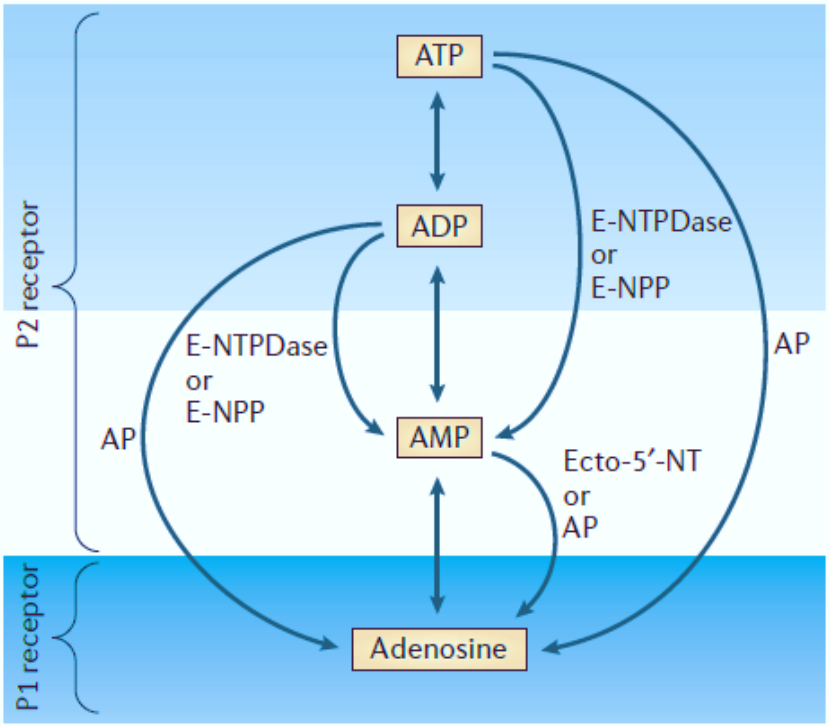


Figura 9- Receptores purinérgicos ligam o ATP extracelular e os produtos das reações que resultam da hidrólise enzimática do ATP por ectonucleotidases. Adaptado de (Fields e Burnstock, 2006).

Após a liberação no meio extracelular e a ativação dos receptores específicos, os nucleotídeos de adenina podem ser hidrolisados, através de sucessivas reações, por ecto-enzimas ou caracteristicamente nomeadas de ectonucleotidases, que convertem estes nucleotídeos até a produção de adenosina. Essa via constitui uma cascata enzimática capaz de controlar a concentração e o tempo em que essas moléculas sinalizadoras permanecem no espaço extracelular (Figura 9) (Zimmermann, 2001).

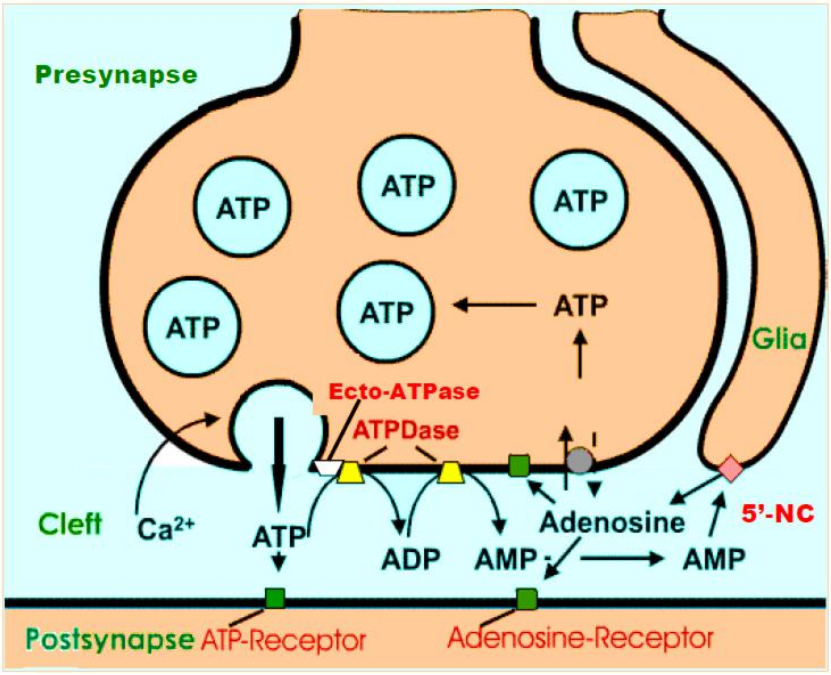


Figura 10. Funções do ATP liberado no terminal nervoso e sua completa hidrólise até adenosina no espaço extracelular (Zimmermann, 2001).

As ectonucleotidases constituem um eficiente mecanismo de controle dos níveis de nucleotídeos e nucleosídeos no espaço extracelular (Zimmermann, 2001). Várias famílias de ectonucleotidases podem degradar os nucleotídeos extracelulares, dentre os quais podemos citar os membros da família das E-NTPDases (Ecto-nucleosídeo trifosfato difosfoidrolases), E-NPPS (Ecto-nucleotídeo pirofosfatase/fosfodiesterases, PDEase, EC 3.1.4.1) e as fosfatases alcalinas, para a hidrolise de nucleotídeos di e trifosfatados. Os nucleotídeos monofosfatados podem ser hidrolisados pela ecto-5'-nucleotidase e fosfatases alcalinas (Figura 11) (Zimmermann, 2001).

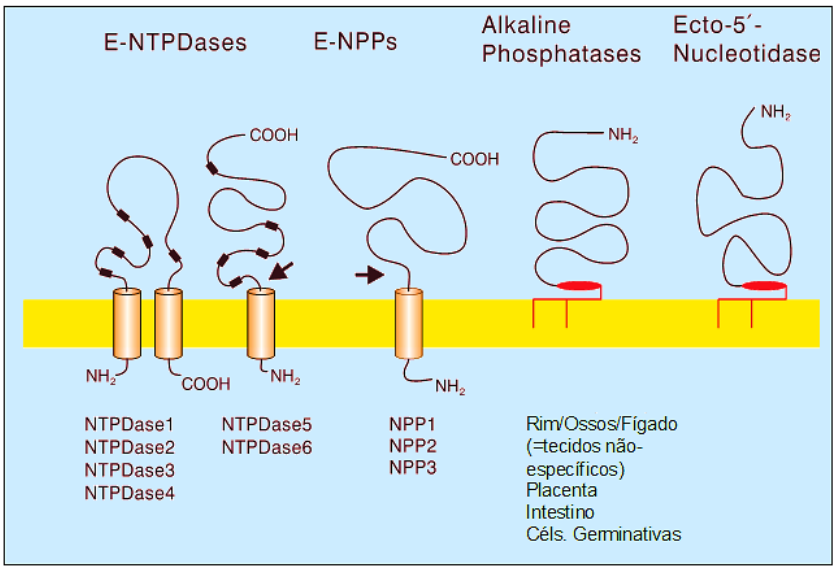


Figura 11. Atividade catalítica e topografia de membrana para a família das ectonucleotidases Adaptado de (Zimmermann, 2001).

Estudos relatam a atividade de membros de uma família de nucleosídeo trifosfato difosfoidrolases como as NTPDases 1 a 8 (E.C. 3.6.1.5), e uma 5'-nucleotidase (E.C. 3.1.3.5, CD73) participarem no controle dos níveis extracelulares do ATP na fenda sináptica e no controle da neuromodulação e da neurotransmissão purinérgica (Robson, Sevigny *et al.*, 2006).

A família das E-NTPDases são enzimas ancoradas à membrana plasmática via domínios hidrofóbicos, com o sitio ativo voltado para o meio extracelular. Estas enzimas são caracterizadas por hidrolisar nucleotídeos extracelulares tri e difosfatados na presença de Ca^{2+} e Mg^{2+} (Robson, Sevigny *et al.*, 2006), sendo bem caracterizada no SNC e em outros tecidos, como em plaquetas e em linfócitos (Schetinger, Vieira *et al.*, 2001; Lunkes, Lunkes *et al.*, 2003). Quatro das NTPDases são enzimas tipicamente localizadas na membrana celular com um sítio catalítico voltado para a face extracelular (NTPDase 1, 2, 3 e 8), enquanto que as quatro restantes exibem localização intracelular (NTPDases 4, 5, 6 e 7) (Robson, Sevigny *et al.*, 2006) como pode ser observado na figura 12, adaptado de Robson e colaboradores (2006):

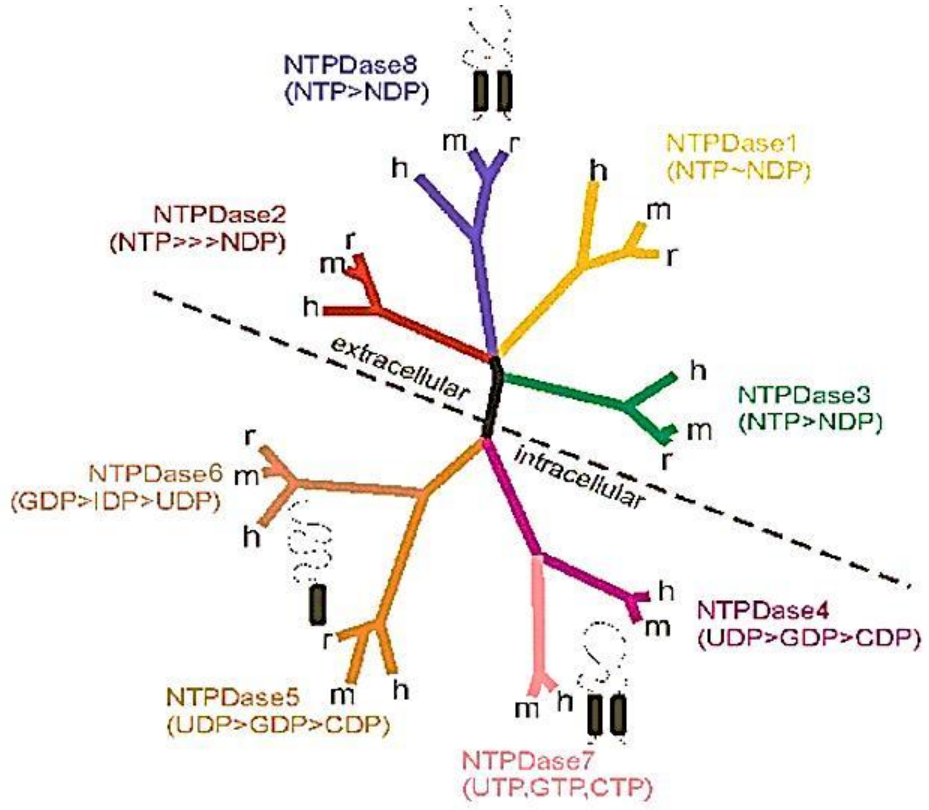


Figura 12. Membros da Família das NTPDases.

As 5'-nucleotidases (EC 3.1.3.5) constituem uma família de enzimas com distribuição tecidual ampla e com capacidade de produzir nucleosídeos a partir de nucleotídeos 5'-monofosfatados (Zimmermann, 1996). Diferentes distribuições celulares são encontradas para a família de 5'-nucleotidases, existindo na forma solúvel e ancoradas à membrana plasmática (Bianchi e Spychala, 2003). A 5'-nucleotidase (5'-NT) é uma glicoproteína ancorada à membrana plasmática através de uma âncora lipídica de glicosilfosfatidilinositol (GPI), com peso molecular aparente de 62 a 74 kDa e que possui forma estrutural de dímero com pontes dissulfeto entre as cadeias (Zimmermann, 2001). Está presente na maioria dos tecidos, sendo expressa na superfície de células nervosas, e nas sinapses durante o seu desenvolvimento e remodelação (Zimmermann, Braun *et al.*, 1998).

O sitio catalítico desta enzima está voltado para o meio extracelular, e sua atividade catalítica é inteiramente ativada por cátions divalentes e inibida por ADP, ATP e 5'-α,β-metileno-difosfato. Sete subtipos de 5'-nucleotidases foram clonados, sendo que seis delas de localização intracelular e uma na membrana plasmática

(Bianchi e Spychala, 2003). O AMP é o nucleotídeo mais eficientemente hidrolisado pela 5'-nucleotidase, mas a enzima pode hidrolisar também CMP, UMP, IMP, GMP, até seus respectivos nucleosídeos (Cunha, 2001). A participação da 5'-NT na via das ectonucleotidases têm um papel primordial na modulação da produção da adenosina extracelular. A adenosina é uma purina endógena com importante papel na regulação da excitabilidade neuronal e na transmissão sináptica de baixa freqüência, sendo também considerada como neuromodulador no fenômeno de plasticidade sináptica (Cunha, 2001).

A atividade da 5'-NT encontra-se aumentada em astrócitos, células microgliais (Bianchi e Spychala, 2003) e em sinaptossomas de hipocampo de ratos após a isquêmica focal e reperfusão (Schetinger, Barcellos *et al.*, 1994). Além disso, foi demonstrado que, em várias regiões cerebrais, a atividade desta enzima é crescente à medida que o animal envelhece (Fuchs, 1991). A atividade da 5'-NT mostrou-se alterada em diferentes modelos experimentais estudados por pesquisadores do nosso grupo, em ratos desmielinizados (Spanevello, Mazzanti *et al.*, 2006) e diabéticos (Schmatz, Schetinger *et al.*, 2009) ocorrendo um aumento na sua atividade em sinaptossomas de córtex cerebral, indicando que esta enzima pode ter um papel crucial no SNC por aumentar os níveis de adenosina extracellular, um reconhecido nucleosídeo com propriedades neuromodulatórias e neuroprotetoras.

A enzima Na^+,K^+-ATPase, também conhecida como bomba de sódio e potássio, é uma proteína ligada à membrana com propriedades catalíticas. Seu papel é transportar ativamente três íons sódio para fora da célula e dois íons potássio para dentro, contra seus gradientes de concentração, consumindo uma molécula de ATP (Skou, 1957; Skou e Esmann, 1992; Kaplan, 2002). Para a realização do transporte, a Na^+,K^+-ATPase apresenta em sua estrutura a subunidade alfa, que contém a porção catalítica, e a subunidades beta, responsável pela inserção da enzima na membrana celular. A porção gama pertence a uma família de proteínas de membrana envolvidas no transporte de íons pela membrana e não apresenta sua função completamente estabelecida (Skou e Esmann, 1992; Kaplan, 2002). A Na^+,K^+-ATPase desempenha um papel vital para o funcionamento e para a sobrevivência das células atuando na manutenção da homeostase dos eletrólitos intracelulares em praticamente todos os tecidos. No sistema nervoso central sua atividade é de extrema importância para a manutenção do gradiente eletroquímico através da membrana plasmática (Lingrel e Kuntzweiler, 1994; Lingrel,

Van Huysse *et al.*, 1994). Este gradiente atua como fonte energética para manutenção do potencial de repouso e da excitabilidade neuronal, bem como na regulação do pH intracelular e no volume da célula (Geering, 1990). Estudos mostram que a Na^+,K^+-ATPase também desempenha um importante papel na plasticidade sináptica, na potencialização de longa duração (Glushchenko e Izvarina, 1997) e no aprendizado e memória em diferentes tarefas (Brunelli, Garcia-Gil *et al.*, 1997; Gibbs, 2003; Wyse, Bavaresco *et al.*, 2004; Zhan, Tada *et al.*, 2004). Quando ocorre a diminuição da atividade desta enzima, a neurotransmissão e a atividade neural são prejudicadas (Lees, Lehmann *et al.*, 1990; Jamme, Petit *et al.*, 1995; Li e Stys, 2001; Vaillend, Mason *et al.*, 2002). Além disso, tem sido descrito que inibidores da atividade da Na^+,K^+-ATPase induzem aumento de Ca2+ intracelular e o estabelecimento de insultos excitotóxicos (Brines e Robbins, 1992; Greene e Greenamyre, 1996; Veldhuis, Van Der Stelt *et al.*, 2003) e pioram a memória em ratos (Rogers, Oettinger *et al.*, 1977; Sato, Tanaka *et al.*, 2004; Zhan, Tada *et al.*, 2004). Tem sido demonstrado que a inibição da atividade enzimática da Na^+,K^+-ATPase está envolvida com o desenvolvimento neurodegenerativas (Kumar e Kurup, 2002). Na doença de Alzheimer, por exemplo, estudos revelam uma diminuição tanto na expressão (Chauhan, Lee *et al.*, 1997) quanto na atividade. desta enzima em humanos (Liguri, Taddei *et al.*, 1990).

A enzima Ca^{2+}-ATPAse também é uma ATPAse ancorada a membrana celular e atua na manutenção das concentrações de Ca^{2+} citoplasmáticas uma vez que promove a extrusão do Ca^{2+} intracelular (Fakira, Gaspers *et al.*, 2012; Lukyanets e Lukyanetz, 2013). Modelos experimentais para a DA tem mostrado que há um influxo excessivo de íons cálcio e aumento de suas concentrações intracelulares (Chin, Tse *et al.*, 2006; Vignini, Nanetti *et al.*, 2007). Além disso, estudos também reportam que uma diferença de afinidade da Ca^{2+}-ATPase para íons Ca^{2+} pode estar relacionado com a etiologia da doença de Alzheimer. (Rizopoulos, Chambers *et al.*, 1988). Foi descoberto que a Ca^{2+}-ATPAse comporta-se de maneira diferente quando ha um aumento no influxo de cálcio em pacientes com Alzheimer que apresentaram uma menor atividade em relação a pacientes que não possuem Alzheimer (Rizopoulos, Chambers *et al.*, 1989).

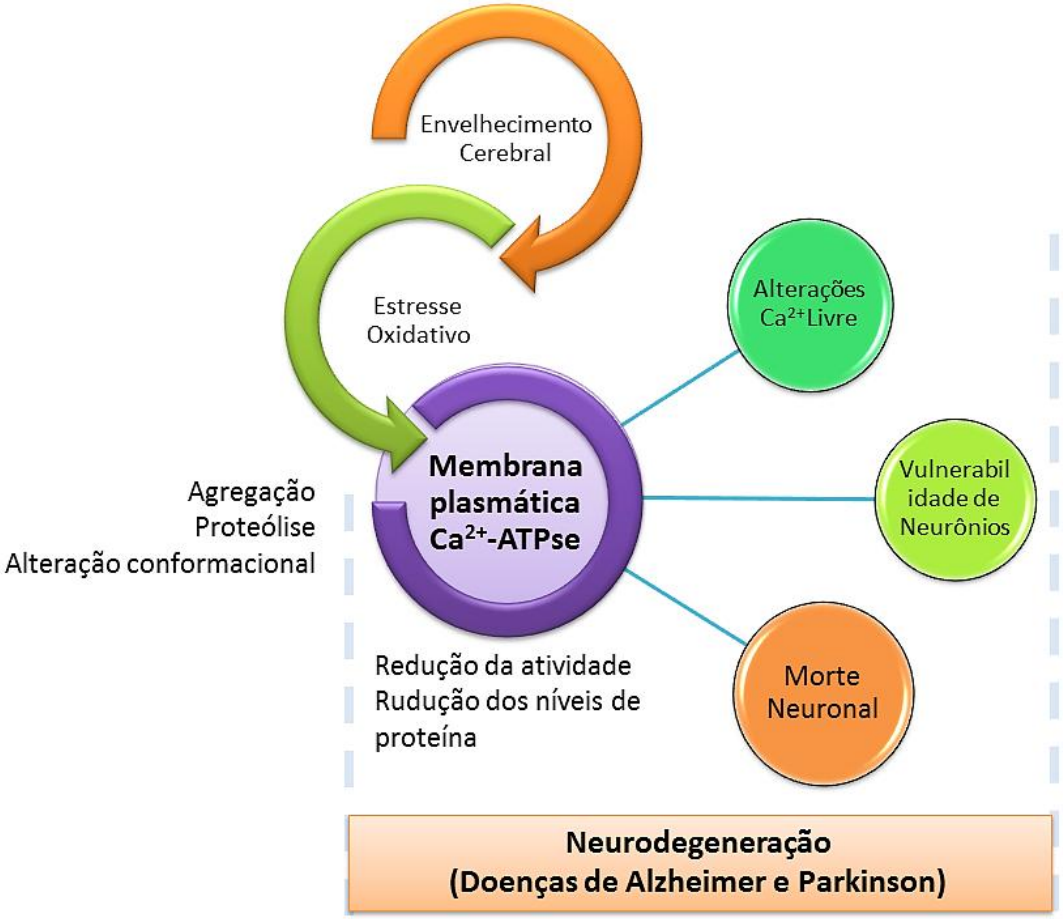


Figura 13. Esquema de eventos celulares relacionados com o envelhecimento cerebral e doenças neurodegenerativas. Adaptado de (Zaidi, 2010)

Estudos com envelhecimento também demonstram uma redução na expressão da regucalcina, uma proteína ligante do cálcio, que regula as concentrações intracelulares de Ca^{2+} e a atividade da Ca^{2+}-ATPase (Yamaguchi, 2012). O envelhecimento cerebral leva a um aumento no estresse oxidativo associado a membrana, que por sua vez prejudica a atividade das enzimas ancoradas a membrana. A redução na atividade destas enzimas, dentre elas a Ca-ATPase reduz o desenvolvimento de neuritos, aumenta a suceptibilidade neuronal ao estresse celular favorecendo o desenvolvimento de doenças neurodegenerativas.

Neste contexto, tendo em vista que tanto a sinalização purinérgica como colinérgica constituem-se atualmente como importantes alvos de estudos que investigam os mecanismos da degeneração mnemônica e a demência no

envelhecimento, torna-se relevante avaliar se compostos com propriedades neuroprotetoras e que melhoram a memória possam regular estes sistemas, a fim de contribuir para a busca de novas terapias que possam beneficiar pacientes com distúrbios cognitivos e demências.

2. Objetivos

2.1 Objetivo geral

O principal objetivo deste estudo foi determinar a capacidade neuroprotetora das antocianinas de prevenirem os déficits de memória em um modelo de amnésia, e déficits cognitivos em um modelo de demência esporádica do tipo Alzheimer, e em seguida definir as antocianinas como compostos que podem regular o sistema purinérgico e colinérgico, de modo a permitir traçar um mecanismo de ação para a neuroproteção conferida pelo consumo de curto prazo de antocianinas.

2.2 Objetivos específicos

- Verificar o efeito do pré-tratamento com antocianinas sobre parâmetros de memória, ansiedade e locomoção em um modelo de amnésia e de demência esporádica do tipo Alzheimer em ratos.
- Determinar o efeito do tratamento com antocianinas sobre a atividade das enzimas acetilcolinesterase, Na^+, K^+-ATPase e Ca^{2+}-ATPase em um modelo de amnésia e de demência esporádica do tipo Alzheimer em ratos.
- Determinar o efeito do tratamento com antocianinas sobre atividade das enzimas NTPDase e 5'-nucleotidase em um modelo de amnésia e de demência esporádica do tipo Alzheimer em ratos.
- Caracterizar alterações na densidade e imunorreatividade para enzimas NTPDase 1 e 5'-nucleotidase em um modelo de demência esporádica do tipo Alzheimer em ratos.
- Avaliar alterações em parâmetros de estresse oxidativo e nitrosativo em um modelo de demência esporádica do tipo Alzheimer em ratos.
- Avaliar, *in vitro*, a afinidade das antocianinas para o sítio benzodiazepínico de receptores $GABA_A$ em membranas sinápticas purificadas.

3. Manuscrito I

3.1 Neuroprotective effects of Anthocyanins on scopolamine-induced amnesia via anti-acetylcholinesterase and membrane bound ATPases in rats

Jessié M. Gutierres[a]*[#], Fabiano B. Carvalho[b#], Maria Rosa C. Schetinger[a], Juliano M. Vieira[a], Patricia C. Marisco[a], Cristiane Signor[a], Maribel A. Rubin[a], Vera M. Morsch[a], Cinthia M. Mazzanti[b], Paula Agostinho[c], Roselia Spanevello[d].

[a] Departamento de Química, Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria, Santa Maria/RS 97105-900, Brasil.

[b] Setor de Bioquímica e Biologia Molecular do Laboratório de Terapia Celular, Centro de Ciências Rurais; Universidade Federal de Santa Maria, Santa Maria/RS 97105-900, Brasil.

[c] Center for Neuroscience and Cell Biology, Faculty of Medicine, Biochemistry Institute, University of Coimbra, 3004 Coimbra, Portugal

[d] Centro de Ciências Químicas, Farmacêuticas e de Alimentos, Universidade Federal de Pelotas, Campus Universitário, Capão do Leão, Pelotas/RS 96010-900, Brasil.

*Corresponding authors:

Jessié Martins Gutierres: Departamento de Química, Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria, Santa Maria/RS 97105-900, Brasil. Tel./fax: + 55-55 3220 9557.

E-mail address: jessiegutierres@hotmail.com (J.M.Gutierres)

Cinthia Melazzo Mazzanti: Setor de Bioquímica e Biologia Molecular do Laboratório de Terapia Celular, Centro de Ciências Rurais; Universidade Federal de Santa Maria, Santa Maria/RS 97105-900, Brasil.

Brasil. Tel./fax: + 55-55 3220 9557.

E-mail address: cmelazzomazzanti@gmail.com (C.M. Mazzanti)

[#] Jessié M Gutierres and Fabiano B. Carvalho equal contribution to this study

Abstract

Anthocyanins are a group of naturally occuring phenolic compounds related to improve memory and have antioxidant properties. Here, we have investigated the therapeutic efficacy of anthocyanins in a pharmacological model for Alzheimer's disease induced by scopolamine. Moreover, we evaluated whether the levels of nitrite/nitrate (NOx), as well as Na^+,K^+-ATPase, Ca^{2+}-ATPase and acethylcholinesterase (AChE) activities in the cerebral cortex (CC) and hippocampus (HC) are altered in scopolamine administered animals. The animals were divided in 4 different groups: control (CTRL), anthocyanins (ANT), scopolamine (SCO), and scopolamine+anthocyanins (SCO+ANT). After seven days of treatment with ANT (200mg/kg; oral), the animals were SCO injected (1mg/kg; IP), and later were performed the behavior parameters, and submitted to euthanasia. A memory deficit was found in SCO group, but ANT treatment showed to prevent this impairment of memory ($P<0.05$). Our results showed a *per se* an anxiolitic effect of ANT treatment ($P<0.05$). AChE activity was found to be increased in HC and CC in SCO group, which was attenuated by ANT ($P<0.05$). SCO decreased Na^+,K^+-ATPase activity in HC and CC, and significant decreased Ca^{2+}-ATPase activity in HC, but ANT was able to prevent these effects ($P<0.05$). NOx levels showed no significant interactions between groups both in HC and CC. In conclusion, this study was a first to show that ANT is able to modulate cholinergic neurotransmission and protect enzymes ATP dependent, as well as act as anxiolytic compound and enhance memory in scopolamine administered animals.

Keywords: Anthocyanins; Scopolamine; Acetylcholinesterase; Memory; Anxiety-like behaviour.

Introduction

Alzheimer's disease (AD) is a degenerative disorder affecting memory, judgment and the ability to reason (Scarpini e Cogiamanian, 2003; Scarpini, Scheltens *et al.*, 2003). AD results from neurodegeneration characterized by the deposition of amyloid plaques, the development of neurofibrillary tangles, inflammation, and neuronal loss in specific regions of the forebrain (Sambamurti, Greig *et al.*, 2002). Although multiple neurotransmitter systems appear to be affected in AD, cholinergic degeneration and functional impairment have received the greatest amount of research interest. Acetylcholinesterase (AChE) is an important enzyme involved in cognitive process of learning and memory since this enzyme rapidly hydrolyses acetylcholine (ACh) promoting the maintenance of these levels in the synaptic cleft (Gron, Brandenburg *et al.*, 2006; Hut e Van Der Zee, 2011). The ACh is a neurotransmitter with a major role in the regulation of cognitive functions that is mainly found in the brain, muscles and cholinergic neurons (Blokland, 1995; Paleari, Grozio *et al.*, 2008). This shows that AChE is not limited to cholinergic transmission but also is implicated in several non-cholinergic actions including cell proliferation (Appleyard, 1994) and neurite outgrowth (Chacon, Reyes *et al.*, 2003). In this way the AChE activity has been the target of the emerging therapeutic strategies to treat cognitive disorders and the use of polyphenols in red wine have been proposed by researchers for these compounds has properties of modulate the affected pathways for neurodegenerative diseases like AD (Ibach e Haen, 2004; Musial, Bajda *et al.*, 2007).

Anthocyanins (ANT) are flavonoid found in grape juice and red wine, with phenolic groups present in their chemical structure and give colors a wide variety of flowers and fruits (Williams e Grayer, 2004; Veitch e Grayer, 2008; Yoshida, Mori *et al.*, 2009). It has been shown that ANT are potent antioxidants (Kahkonen, Hopia *et al.*, 2001; Kahkonen e Heinonen, 2003) and have neuroprotective properties (Del Rio, Borges *et al.*, 2010), preventing deleterious effects found in models of Parkinson's (Kim, Ju *et al.*, 2010) and AD disease (Shih, Chan *et al.*, 2010). It can suggest that flavonoids compounds also have beneficial effects on memory and cognition (Spencer, 2010) since ANT improves memory in old rats in Morris water maze (Andres-Lacueva, Shukitt-Hale *et al.*, 2005) and also in older humans (Krikorian, Shidler *et al.*, 2010).

Cognitive disorders affect the activity of Na^+,K^+-ATPase and Ca^{2+}-ATPase, important enzymes that contribute for ionic homeostase, generation of the membrane potential and synaptic neurotransmission. Na^+,K^+-ATPase is responsible for the active transport of Na^+ and K^+ and maintains the ionic gradient for neuronal excitability (Kaplan, 2002; Jorgensen, Hakansson *et al.*, 2003). Moreover, Na^+,K^+-ATPase might play a relevant role in neuronal and synaptic plasticity (Glushchenko e Izvarina, 1997; Scuri, Lombardo *et al.*, 2007) and decreased enzyme activity or expression directly impairs signaling, with deleterious consequences on memory and anxiety in rats (Dos Reis, De Oliveira *et al.*, 2002; Moseley, Williams *et al.*, 2007), increases Ca^{2+} influx in brain slices (Fujisawa, Kajikawa *et al.*, 1965) and causes death in rats (Lees, Lehmann *et al.*, 1990). Ca^{2+}-ATPase is responsible for control of intracellular Ca^{2+} homeostasis. Furthermore, the decreased activity of Ca^{2+}-ATPase has been associated with production of reactive oxygen species and neurodegenerative diseases (Skou e Esmann, 1992; Kodavanti, 1999; Clarke e Fan, 2011).

Scopolamine (SCO) is a non-selective muscarinic antagonist used to induce deficits in animal models of memory dysfunction (Klinkenberg e Blokland, 2010). It has been reported also that SCO reduces frontal cortex perfusion in young humans (Honer, Prohovnik *et al.*, 1988) and impairs the energetic metabolism reducing the ATP levels in the cerebral cortex of rats (Ray, Blin *et al.*, 1992; Blin, Piercey *et al.*, 1994). The impairment of mitochondrial function and reduction of ATP levels are pathological conditions found in neurodegenerative diseases such as AD, which is closely linked to the decline of cognitive processes (Ferrer, 2009; Hauptmann, Scherping *et al.*, 2009).

In this context, since ANT has an important function as antioxidant andneuroprotective compound, in this study we investigated whether this natural compound has the ability to prevent memory deficits found in a mnemonic model induced by SCO. We also evaluated the nitrite/nitrate (NOx) levels, as well as the activities of enzymes important for neurotransmission such as AChE, Na^+,K^+-ATPase and Ca^{2+}-ATPase, which are known to be altered in Alzheimer's disease.

Results

Behavioral tests

Anthocyanins prevents the impairment of memory induced by scopolamine.

In this study we used 4 groups of animals: control (CTRL), anthocyanins (ANT), scopolamine (SCO), and scopolamine plus anthocyanins (SCO+ANT). Table 1 shows the effect of the treatment with ANT on the SCO-induced memory deficits, in the step-down latencies. Statistical analysis of Scheirer–Ray–Hare test (*nonparametric two-way ANOVA*) showed a significant saline or SCO (1mg/kg; IP) *vs* saline or ANT (200mg/kg) interaction, revealing that treatment with ANT prevented the impairment of memory induced by SCO (Table 1). Statistical analysis of training showed no difference between groups (Table 1). However, motivational disparities in the training session may account for differences in inhibitory avoidance at testing, experiments were performed to assess whether SCO or ANT affected shock threshold, or locomotor ability of the animals. Statistical analysis of open-field data (*one-way ANOVA*) revealed that SCO did not alter the number of crossing [$F(3,36)=0.99$, $P>0.05$; Table 2] or rearing [$F(3,36)=0.13$, $P>0.05$; Table 2] responses in a subsequent open-field test session, suggesting that neither SCO nor ANT caused gross motor disabilities at testing. Moreover, SCO did not alter foot shock sensitivity, as demonstrated by the similar flinch and jump thresholds exhibited by the animals. These data suggest that neither treatment with SCO+ANT administered before nor SCO administered after training of inhibitory avoidance caused motor disabilities or altered foot shock sensitivity: flinch [$F(3,36)= 1.30$; $P>0.05$], jump [$F(3,36)= 0.48$; $P>0.05$] and vocalization [$F(3,36)= 1.11$; $P>0.05$] (Table 2).

Effect of anthocyanins treatment on anxiolytic-like behavior

Although there are studies showing that flavonoids have anxiolytic effects, there are no studies showing that ANT act as compounds possessing these properties. In this sense, we decided to investigate the effect of the ANT or SCO treatments on anxiolytic-like behavior in the elevated plus maze task (Figure 1). Statistical analysis of testing (*two-way ANOVA*) showed a significant Saline or ANT (200 mg/kg) interaction to Time in Closed Arms [$F(1,36)= 14.780$; $P<0.0001$; Figure 1B], revealing that treatment with ANT has a *per se*

anxiolytic effect. Did not observed significant difference between ANT or SCO treatments on % Time in Open Arms [F (1,36)= 0.001; *P>0.05;* Figure 1A] and Nº Entries in Closed Arms [F (1,36)= 0.132; *P>0.05;* Figure 1C] or Nº Entries in Arms [F (1,36)= 0.846; *P>0.05;* Figure 1D].

Enzymatic activities

Anthocyanins prevents the increase in AChE activity induced by scopolamine.

The evidence pointing to cholinergic impairments come from studies that report alterations in AChE activity, the sequence of experiments we investigated whether ANT restores AChE activity in the pharmacological model of AD induced by SCO. Figure 2 shows the effect of ANT and SCO on the activity of AChE in cerebral cortex and hippocampus, both in S1 and synaptosomes of rats. Statistical analysis of testing (*two-way ANOVA*) showed a significant Saline or SCO (1mg/kg) *vs* Saline or ANT (200m/kg) interaction, suggesting that the ANT treatment prevents the increase in AChE activity in synaptosomes of cerebral cortex [F= (1,28)= 6.135; *P<0.05;* Figure 2A] and hippocampus [F= (1,28)= 7.515; *P<0.05;* Figure 2A] induced by SCO.

Statistical analysis of testing (*two-way ANOVA*) also showed a significant Saline or SCO (1mg/kg) *vs* Saline or ANT (200mg/kg) interaction, suggesting that the ANT treatment prevents the increase in AChE activity in S1 fraction of cerebral cortex [F= (1,28)= 6.322; *P<0.05;* Figure 2B] and hippocampus [F (1,28)= 5. 447; *P<0.05;* Figure 2B] induced by SCO.

Anthocyanins prevents the decrease of Na^+,K^+-ATPase and Ca^{2+}-ATPase activities induced by scopolamine in hippocampus.

Na^+,K^+-ATPase and Ca^{2+}-ATPase are enzymes involved in the control of neurotransmission, since regulating membrane potential and intracellular Ca^{2+}concentrations, respectively. Figure 3 shows the effect of ANT and SCO on the activity of Na^+,K^+-ATPase and Ca^{2+}-ATPase in cerebral cortex and hippocampus of rats. Statistical analysis of testing (*two-way ANOVA*) showed a significant Saline or SCO (1mg/kg) *vs* Saline or ANT (200mg/kg) interaction, suggesting that the ANT treatment prevents the decrease in Na^+,K^+-ATPase activity in cerebral cortex [F (1,28)= 7.781; P<0.05] and hippocampus [F (1,28)= 5.866; P<0.05] induced by SCO (Figure 3).

Additionally, *two-way ANOVA* showed a significant Saline or SCO (1mg/kg) *vs* Saline or ANT (200mg/kg) interaction, suggesting that the ANT treatment also prevents the decrease of Ca^{2+}-ATPase activity in the hippocampus [F (1,28)= 4.803; P<0.05] induced by SCO (Figure 3B). However, in cerebral cortex did not observed significant differences between groups [F (1,28)= 1.080, P<0.05]

NOx levels determination

Anthocyanins are described to possess antioxidant effects, at this set of experiments we decided to investigate if ANT alters nitrite plus nitrate (NOx) in the brain of rats. Figure 4 shows the effect of ANT and SCO on the NOx levels production in cerebral cortex and hippocampus of rats. Statistical analysis of testing (*two-way ANOVA*) showed no significant interactions between groups in cerebral cortex [F (1,28)= 1.149; *P<0.05*] and hippocampus [F (1,28)= 0.009; *P<0.05*]

Discussion

Ageing has been recognized as an irreversible and inevitable process since ancient times. Ageing-associated disorders include immune dysfunction (Candore, Balistreri *et al.*, 2006; Sansoni, Vescovini *et al.*, 2008), cognition degeneration (Barzilai, Atzmon *et al.*, 2006; Mehta, 2007), cardiovascular disease (Dominguez e Barbagallo, 2007) and metabolic syndrome (Maggi, Noale *et al.*, 2008). Increasing evidence suggests that ageing increases the risk of degeneration of the nervous system, which mostly affects the moral and physiological life of the elderly. As a result of the development of medical science and health care, the average human life span is increasing; however, the future socioeconomic burden of the elderly must be a source of concern in developed countries (Shih, Chan *et al.*, 2010).

A number of investigators have found that flavonoids, including some anthocyanins, possess oral bioavailability in rats (Miyazawa, Nakagawa *et al.*, 1999; Matsumoto, Inaba *et al.*, 2001; Mcghie, Ainge *et al.*, 2003) and that they are able to cross the rat blood–brain barrier after blueberry (Andres-Lacueva, Shukitt-Hale *et al.*, 2005) and blackberry (Talavera, Felgines *et al.*, 2005) supplementation, as well as after a single administration (Youdim, Dobbie *et al.*,

2003; Passamonti, Vrhovsek *et al.*, 2005) suggesting that these compounds can feasibly have a direct effect on brain processes. Dietary consumption in some individuals has been estimated to be up to 200 mg/day of anthocyanins, which is higher than that of other flavonoids (23 mg/day) such as quercetin (Scalbert e Williamson, 2000; Frank, Kamal-Eldin *et al.*, 2002; Mcghie, Ainge *et al.*, 2003). In the present study, pre-administration of anthocyanins (ANT) potentiated memory retention in scopolamine (SCO) administered animals, and evidences have demonstrate that ANT is able to improve memory of old rats in Morris water maze (Andres-Lacueva, Shukitt-Hale *et al.*, 2005), and of mice in the inhibitory avoidance task (Barros, Amaral *et al.*, 2006) and elderly humans (Krikorian, Shidler *et al.*, 2010). Moreover, a 2-month dietary supplementation of rats with blueberries prevented deficits in learning performance induced by bilateral hippocampal injections of kainic acid, and the loss of CA1 pyramidal neurons (Duffy, Spangler *et al.*, 2008). It has been shown that ANT are potent antioxidants, and prove to be effective scavengers of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Kahkonen, Hopia *et al.*, 2001; Kahkonen e Heinonen, 2003), having a clear neuroprotective role (Del Rio, Borges *et al.*, 2010).

These results implicate that ANT possess health benefits. Of particular interest, procyanidins as well as resveratrol are considered to be one of the bioactive ingredients in red wine for the cardioprotective effects, known as “French Paradox” (Nishizuka, Fujita *et al.*, 2011). If this is the case, these protective effects conferred on polyphenols in red wine also may be related to prevention of learning and memory deficits associated with aging, since it is well recognized that populations which consume rich fruit anthocyanins have an improvement in memory (Krikorian, Eliassen *et al.*, 2010; Krikorian, Boespflug *et al.*, 2012).

Furthermore, shock motivated learning tests, particularly in those that investigate the effect of drugs given before the acquisition test, is whether pharmacological treatments affect locomotor activities or motivational aspects of learning, such as shock sensitivity. Immediately after inhibitory avoidance test, the animals were subjected to an open-field test which is widely used for evaluating motor abnormalities (Belzung e Griebel, 2001). The open field session revealed that the treatment with SCO or ANT did not alter spontaneous locomotor activity, the animals showed a similar number of crossing or rearing

responses (Table 2). Moreover, we observed that the rats of different groups did not shows altered shock sensitivity, as verified by their similar flinch, jump and vocalization thresholds (Table 2). Thus, these data suggest that neither SCO 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 to learning and memory evaluation, we also measured the anxiolytic-like behavior of the rats by the elevated plus maze task, commonly used to study anxiety-related behavior in rodents (Belzung e Griebel, 2001). Our result showed an anxiolytic effect *per se* of ANT group (200 mg/kg) (Figure 1 B). Thus, these findings are in agreement with other studies which show that ANT also has an anxiolytic effects in rats and mice in the elevated-plus maze test (Ramirez, Izquierdo *et al.*, 2005; Barros, Amaral *et al.*, 2006). Our research group investigated whether ANT has affinity for $GABA_A$ receptors (data not shown), important targets for the control of anxiety, and in this study the ANT (100µM) was shown to have affinity for $GABA_A$ receptors since that displacement about 60% binding of flunitrazepan to the benzodiazepine site of the $GABA_A$ receptor.

The activation of muscarinic m1 receptors, which are coupled to the phosphoinositide (PI) second messenger transduction system, is the initial objective of cholinergic replacement therapy in AD (Bymaster, Carter *et al.*, 1998; Bymaster, Shannon *et al.*, 1998). This way, scopolamine is used since it compromises cholinergic neurotransmission and, in some ways, mimics the memory deficit observed in diseases characterized by impairment in cholinergic neurotransmission, such as AD (Kopelman e Corn, 1988; Wesnes, Simpson *et al.*, 1991; Christensen, Maltby *et al.*, 1992). The present study showed that ANT attenuated scopolamine-induced impairment on memory retention, indicating that ANT and cholinergic system have a close interaction. The data are in agreement with results of others (Izquierdo, 1989; Blitzer, Gil *et al.*, 1990), which showed that muscarinic cholinoceptors play important roles in hippocampal-based learning, memory and neuronal plasticity (Messer, Bohnett *et al.*, 1990; Anagnostaras, Josselyn *et al.*, 2000). It should be considered that ANT might have a neuroprotective effect in the hippocampal cholinergic system.

Our results showed that scopolamine administration significantly increases AChE activity in the cerebral cortex and hippocampus of animals, and

these results are consistent with other (Jeong, Lee *et al.*, 2008; Choi, Lee *et al.*, 2012; Rang Oh, Jin Kim *et al.*, 2012). Scopolamine has been used to screen antiamnesic drugs for age-related central nervous system (CNS) dysfunction (Sakurai, Kato *et al.*, 1998). The elevation of brain oxidative status after administration of amnesic doses of scopolamine further substantiates the value of scopolamine-induced amnesia as an animal model to test for drugs with potential therapeutic benefits in dementia (El-Sherbiny, Khalifa *et al.*, 2003). In addition, the axonal transport of endogenous AChE showed impairment both of fast antero and retrograde transport (Southam, Thomas *et al.*, 1991). In vivo investigation of rats treated with scopolamine, showed that brain AChE was markedly reduced (Southam, Thomas *et al.*, 1991). Our results showed that scopolamine increased the AChE activity and this effect was prevented by the treatment with ANT. These results together with the parameters of memory seem to show that ANT may exert mechanisms of up-regulation of the cholinergic pathway.

AChE metabolizes ACh to choline and acetyl-CoA. AChE exists into different molecular forms, which can be distinguished on the basis of their shapes, e.g., collagen-tailed asymmetric forms and globular (G) forms (Lane, Potkin *et al.*, 2006). Although few studies have shown that isoforms of AChE may be more expressed in different brain regions (Malatova, Nicak *et al.*, 1980; Zakut, Matzkel *et al.*, 1985; Lane, Potkin *et al.*, 2006), and that these isoforms can be considered important markers for AD (Kasa, Rakonczay *et al.*, 1997; Shen, 2004; Lane, Potkin *et al.*, 2006), it is known that AChE activity in S1 shows the total AChE activity (different isoforms associated), while the synaptosomal are re-sealed nerve terminal with a greater amount of membrane-bound isoforms G4 (Mazzanti, Spanevello *et al.*, 2006). In this case, we found that SCO treatment increase AChE activity both in homogenate (S1) and synaptosomes of cerebral cortex and hippocampus of rats suggesting that all AChE isoforms are altered.

Studies have shown that SCO impairs energy metabolism and reduces the ATP levels in the cerebral cortex of rats (Ray, Blin *et al.*, 1992; Blin, Piercey *et al.*, 1994). It is known that worsening of mitochondrial function and reduction of ATP levels are pathological conditions found in neurodegenerative diseases such as AD, which is closely linked to the decline of cognitive processes (Ferrer, 2009; Hauptmann, Scherping *et al.*, 2009). Other studies also show that

SCO reduces the frontal cortex perfusion in young humans (Honer, Prohovnik *et al.*, 1988). In addition, it was also observed that intramuscular SCO administration impairs the oxygen consumption and the tissue metabolism of the cardiovascular and CNS of humans (Kirvela, Kanto *et al.*, 1994). Corroborating with previous studies Stone et al (1991) found that glucose treatment was able to prevent deficits on the memory induced by SCO, suggesting that deleterious effects of SCO could be related to energy depletion in neurons (Stone, Walser *et al.*, 1991). In addition, our group showed that SCO reduced the levels of ATP in the cerebral cortex and hippocampus of rats, and ANT treatment prevented the depletion of ATP tissue in both structures (Gutierres, J. M., Carvalho, F. B. *et al.*, 2012). To explain this effect, the possible mechanism could be related to the vasodilatory capacity of anthocyanins (Mudnic, Budimir *et al.*, 2011), since that ANT crosses the blood brain barrier (Youdim, Dobbie *et al.*, 2003), induces vasodilation and activate endothelial oxide nitric synthase, thus it increases the production of nitric oxide (Edirisinghe, Banaszewski *et al.*, 2011; Min, Yu *et al.*, 2011; Mudnic, Budimir *et al.*, 2011).

The ATP levels into the cell has been suggested as an important modulator of Na^+,K^+-ATPase and Ca^{2+}-ATPase activities since a reduction of intracellular ATP decreases the activity of these enzymes (Michaelis, Michaelis *et al.*, 1983; Therien e Blostein, 2000; Erecinska e Silver, 2001; Parsons, Sun *et al.*, 2004). The high cost energetic used by these enzymes is responsible to maintain the electrochemical gradient necessary for excitability neuronal, adjustment of cell volume, osmotic balance, transport of molecules attached to the co-transport of Na^+ and intracellular Ca^{2+} homeostasis (Kaplan, 2002; Jorgensen, Hakansson *et al.*, 2003; Mata e Sepulveda, 2010).

Besides alterations in the cholinergic transmission, cognitive disorders also have an impairment of the generation of membrane potential and the influx of neuronal Ca^{2+} (Berrocal, Marcos *et al.*, 2009; Mata, Berrocal *et al.*, 2011). Considering that Na^+,K^+-ATPase is one of the most abundant brain enzyme, consuming about 40–60% of the ATP generated (Kaplan, 2002), it is not surprising that alterations in its activity may cause a variety of abnormalities. It has been describe that a decrease in Na^+,K^+-ATPase results in depletion of intracellular K^+, accumulation of intracellular Na^+, and, consequently, leads to membrane depolarization and increases in intracellular free Ca^{2+} due to

activation of voltage-gated Ca^{2+} channels and a reversed operation of the Na^{+}/Ca^{2+} exchanger (Archibald e White, 1974; Dipolo e Beauge, 1991; Geering, 1997; Pavlov e Sokolov, 2000; Xiao, Wei *et al.*, 2002). On the other hand, alterations in the intracellular Ca^{2+} concentrations are responsible for modulating the activity of Ca^{2+}-ATPase enzyme which regulates the intracellular levels of this second messenger (Mata e Sepulveda, 2010; Verkhratsky, Rodriguez *et al.*, 2012; Yamaguchi, 2012).

We have found a reduction in the activity of Na^{+},K^{+}-ATPase and Ca^{2+}-ATPase activities in cerebral cortex and hippocampus of animals treated with SCO. These enzymes are sensitivities to tissue levels of ATP, it is possible that the decreased of Na^{+},K^{+}-ATPase and Ca^{2+}-ATPase activities induced by SCO may also be associated with the reduction of ATP levels. In line with this view, reduced activity of the Na^{+},K^{+}-ATPase and Ca^{2+}-ATPase has been suggested to play a central role in memory process (Dos Reis, De Oliveira *et al.*, 2002; Lingrel, Williams *et al.*, 2007; Moseley, Williams *et al.*, 2007) and pathogenesis of neurodegenerative diseases, such as AD (Hattori, Kitagawa *et al.*, 1998; Mata, Berrocal *et al.*, 2011) and Parkinson's disease (Grisar, Guillaume *et al.*, 1992; Rose e Valdes, 1994; Zaidi, 2010).

Material and Methods

Chemicals

Acetylthiocholine, Trizma Base, Acetonitrile, Percoll, Coomassie Brilliant Blue G and Scopolamine (SCO) were purchased from Sigma Chemical Co (St Luis, MO, USA). Anthocyanins was extracted and purified from grape skin (AC-12-R-WS-P/10120/Gin:601412) 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 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 NIH Guide for Care and Use of Laboratory Animals, and Brazilian Society for Neuroscience and Behavior (SBNeC) recommendations for animal care. This work was approved by the ethical committee of Federal University of Santa Maria (23081.003601/2012-63).

Drug administration

The animals were divided into two groups of analysis; the first analysis consisted in treat seven to ten animals per group with anthocyanins per 7 days (200mg/kg body weight; by gavage around 10 a.m) and last day the animals received anthocyanins 30 min before the training in inhibitory avoidance apparatus. Scopolamine (1mg/kg) was dissolved in saline and injected intraperitoneally (i.p) 30 min after the training in inhibitory avoidance apparatus in according with previously describe (Ali e Arafa, 2011); the second group of analysis the animals were submitted to same treatment and the euthanasia was two hours post training with seven animals per group (see Scheme 1). The dose of anthocyanins was chosen on the basis of previous studies indicating neuroprotection (Saija, Princi *et al.*, 1990; Manach, Scalbert *et al.*, 2004; Varadinova, Docheva-Drenska *et al.*, 2009; Gutierres, J. M., Carvalho, F. B. *et al.*, 2012). In addition, the daily intake of anthocyanins in residents of the United States is estimated to be about 200 mg or about 9-fold higher than that of other dietary flavonoids, and this also served as a basis for this study (Manach, Scalbert *et al.*, 2004; Wang e Stoner, 2008).

Behavioral procedure

Inhibitory avoidance task

The final day of treatment with anthocyanins (7^{th} days), animals were subjected to training in a step-down inhibitory avoidance apparatus with previously describe (Rubin, Boemo *et al.*, 2000), following the animals received scopolamine (1 mg/kg; IP) thirty minutes after training. Next, twenty four hours after training the animals were subjected to test in a step-down inhibitory avoidance task. Briefly, the rats were subjected to a single 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 rat was 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 300s 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.

Elevated plus maze task

Anxiolytic-like behavior was evaluated using the task of the elevated plus maze as previously described (Frussa-Filho, Barbosa-Junior *et al.*, 1999; Rubin, Albach *et al.*, 2000). The apparatus consists of a wooden structure raised to 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 an open arm. The animal had 5 minutes to explore the apparatus, and the time spent and the number of entries in open and closed-arms were recorded. The apparatus was thoroughly cleaned with 30% ethanol between each session.

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 (Rubin, Albach *et al.*, 2000; Berlese, Sauzem *et al.*, 2005). The animals were placed on the grid and allowed a 3 min habituation period before the start of a series of shocks (1s) 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

The animals were anesthetized under halothane atmosphere before being killed by decapitation and brain were removed and separated into cerebral cortex and hippocampus and placed in a solution of Tris–HCl 10mM, pH 7.4, on ice (Gutierres, Kaizer *et al.*, 2012a). The brain structures were gently homogenized in a glass potter in Tris–HCl solution. Aliquots of resulting brain structure homogenates were stored at −80°C until utilization. Protein was determined previously in a strip that varied for each structure: cerebral cortex (0.7 mg/ml) and hippocampus (0.8 mg/ml), as determined by the Coomassie blue method as previously described (Bradford, 1976), using bovine serum albumin as standard solution.

Synaptosomes Preparation

Synaptosomes were isolated essentially as previously described (Nagy e Delgado-Escueta, 1984), using a discontinuous Percoll gradient. The cerebral cortex, hippocampus and 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 1,000xg 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 0º-4º throughout the procedure and used to measure AChE activity.

Assay of Lactate Desydrogenase (LDH)

The integrity of the synaptosomes preparations was confirmed by determining 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, Brasil).

Determination of AChE activity in brain

The AChE enzymatic assay was determined by a modification of the spectrophotometric method (Gutierres, Jessié Martins, Carvalho, Fabiano Barbosa *et al.*, 2012) as previously described (Ellman, Courtney *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 2min 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.

Na^+,K^+-ATPase activity measurement

Na^+,K^+-ATPase activity was measured as previously described (Carvalho, Mello *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 37ºC, 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 e 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, Hinds *et al.*, 1993) with minor modifications (Trevisan, Maldaner *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 37ºC, 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 e 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.

Assay of NOx (NO_2 plus NO_3) as a marker of NO synthesis

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

Statistical analysis

Statistical analysis of test step-down latencies was carried out by the Scheirer–Ray–Hare extension of the Kruskal–Wallis test (nonparametric two-way ANOVA). The training latency, open field, binding assay and foot shock sensitivity were analyzed by *one-way ANOVA* following 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.

Conclusion

In conclusion, the present study provides evidences suggesting that ANT may affects sensitivity of cholinoreceptors and protect enzymes ATP dependent. Therefore, ANT indeed has a close interaction with the cholinergic system and underlying memory retention process.

Conflicts of Interest statement

There are no conflicts of interest.

Acknowledgements

This study was supported by Christian Hansen LTDA, Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Rio Grande do Sul (FAPERGS) and Fundação Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Brazil.

References

Ali, E.H., Arafa, N.M., 2011. Comparative protective action of curcumin, memantine and diclofenac against scopolamine-induced memory dysfunction. Fitoterapia. 82**,** 601-8.

Anagnostaras, S.G., Josselyn, S.A., Frankland, P.W., Silva, A.J., 2000. Computer-assisted behavioral assessment of Pavlovian fear conditioning in mice. Learn Mem. 7**,** 58-72.

Andres-Lacueva, C., Shukitt-Hale, B., Galli, R.L., Jauregui, O., Lamuela-Raventos, R.M., Joseph, J.A., 2005. Anthocyanins in aged blueberry-fed rats are found centrally and may enhance memory. Nutr Neurosci. 8**,** 111-20.

Appleyard, M.E., 1994. Non-cholinergic functions of acetylcholinesterase. Biochem Soc Trans. 22**,** 749-55.

Archibald, J.T., White, T.D., 1974. Rapid reversal of internal Na+ and K+ contents of synaptosomes by ouabain. Nature. 252**,** 595-6.

Barros, D., Amaral, O.B., Izquierdo, I., Geracitano, L., do Carmo Bassols Raseira, M., Henriques, A.T., Ramirez, M.R., 2006. Behavioral and

genoprotective effects of Vaccinium berries intake in mice. Pharmacol Biochem Behav. 84**,** 229-34.

Barzilai, N., Atzmon, G., Derby, C.A., Bauman, J.M., Lipton, R.B., 2006. A genotype of exceptional longevity is associated with preservation of cognitive function. Neurology. 67**,** 2170-5.

Belzung, C., Griebel, G., 2001. Measuring normal and pathological anxiety-like behaviour in mice: a review. Behav Brain Res. 125**,** 141-9.

Berlese, D.B., Sauzem, P.D., Carati, M.C., Guerra, G.P., Stiegemeier, J.A., Mello, C.F., Rubin, M.A., 2005. Time-dependent modulation of inhibitory avoidance memory by spermidine in rats. Neurobiol Learn Mem. 83**,** 48-53.

Berrocal, M., Marcos, D., Sepulveda, M.R., Perez, M., Avila, J., Mata, A.M., 2009. Altered Ca2+ dependence of synaptosomal plasma membrane Ca2+-ATPase in human brain affected by Alzheimer's disease. FASEB J. 23**,** 1826-34.

Blin, J., Piercey, M.F., Giuffra, M.E., Mouradian, M.M., Chase, T.N., 1994. Metabolic effects of scopolamine and physostigmine in human brain measured by positron emission tomography. J Neurol Sci. 123**,** 44-51.

Blitzer, R.D., Gil, O., Landau, E.M., 1990. Cholinergic stimulation enhances long-term potentiation in the CA1 region of rat hippocampus. Neurosci Lett. 119**,** 207-10.

Blokland, A., 1995. Acetylcholine: a neurotransmitter for learning and memory? Brain Res Brain Res Rev. 21**,** 285-300.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 72**,** 248-54.

Bymaster, F.P., Carter, P.A., Peters, S.C., Zhang, W., Ward, J.S., Mitch, C.H., Calligaro, D.O., Whitesitt, C.A., DeLapp, N., Shannon, H.E., Rimvall, K., Jeppesen, L., Sheardown, M.J., Fink-Jensen, A., Sauerberg, P., 1998a. Xanomeline compared to other muscarinic agents on stimulation of phosphoinositide hydrolysis in vivo and other cholinomimetic effects. Brain Res. 795**,** 179-90.

Bymaster, F.P., Shannon, H.E., Rasmussen, K., Delapp, N.W., Mitch, C.H., Ward, J.S., Calligaro, D.O., Ludvigsen, T.S., Sheardown, M.J., Olesen, P.H., Swedberg, M.D., Sauerberg, P., Fink-Jensen, A., 1998b.

Unexpected antipsychotic-like activity with the muscarinic receptor ligand (5R,6R)6-(3-propylthio-1,2,5-thiadiazol-4-yl)-1-azabicyclo[3.2.1]octane. Eur J Pharmacol. 356**,** 109-19.

Candore, G., Balistreri, C.R., Listi, F., Grimaldi, M.P., Vasto, S., Colonna-Romano, G., Franceschi, C., Lio, D., Caselli, G., Caruso, C., 2006. Immunogenetics, gender, and longevity. Ann N Y Acad Sci. 1089**,** 516-37.

Carvalho, F.B., Mello, C.F., Marisco, P.C., Tonello, R., Girardi, B.A., Ferreira, J., Oliveira, M.S., Rubin, M.A., 2012. Spermidine decreases Na(+),K(+)-ATPase activity through NMDA receptor and protein kinase G activation in the hippocampus of rats. Eur J Pharmacol. 684**,** 79-86.

Chacon, M.A., Reyes, A.E., Inestrosa, N.C., 2003. Acetylcholinesterase induces neuronal cell loss, astrocyte hypertrophy and behavioral deficits in mammalian hippocampus. J Neurochem. 87**,** 195-204.

Choi, D.Y., Lee, Y.J., Lee, S.Y., Lee, Y.M., Lee, H.H., Choi, I.S., Oh, K.W., Han, S.B., Nam, S.Y., Hong, J.T., 2012. Attenuation of scopolamine-induced cognitive dysfunction by obovatol. Arch Pharm Res. 35**,** 1279-86.

Christensen, H., Maltby, N., Jorm, A.F., Creasey, H., Broe, G.A., 1992. Cholinergic 'blockade' as a model of the cognitive deficits in Alzheimer's disease. Brain. 115 (Pt 6)**,** 1681-99.

Clarke, R.J., Fan, X., 2011. Pumping ions. Clin Exp Pharmacol Physiol. 38**,** 726-33.

Del Rio, D., Borges, G., Crozier, A., 2010. Berry flavonoids and phenolics: bioavailability and evidence of protective effects. Br J Nutr. 104 Suppl 3**,** S67-90.

DiPolo, R., Beauge, L., 1991. Regulation of Na-Ca exchange. An overview. Ann N Y Acad Sci. 639**,** 100-11.

Dominguez, L.J., Barbagallo, M., 2007. The cardiometabolic syndrome and sarcopenic obesity in older persons. J Cardiometab Syndr. 2**,** 183-9.

dos Reis, E.A., de Oliveira, L.S., Lamers, M.L., Netto, C.A., Wyse, A.T., 2002. Arginine administration inhibits hippocampal Na(+),K(+)-ATPase activity and impairs retention of an inhibitory avoidance task in rats. Brain Res. 951**,** 151-7.

Duffy, K.B., Spangler, E.L., Devan, B.D., Guo, Z., Bowker, J.L., Janas, A.M., Hagepanos, A., Minor, R.K., DeCabo, R., Mouton, P.R., Shukitt-Hale, B.,

Joseph, J.A., Ingram, D.K., 2008. A blueberry-enriched diet provides cellular protection against oxidative stress and reduces a kainate-induced learning impairment in rats. Neurobiol Aging. 29**,** 1680-9.

Edirisinghe, I., Banaszewski, K., Cappozzo, J., McCarthy, D., Burton-Freeman, B.M., 2011. Effect of black currant anthocyanins on the activation of endothelial nitric oxide synthase (eNOS) in vitro in human endothelial cells. J Agric Food Chem. 59**,** 8616-24.

El-Sherbiny, D.A., Khalifa, A.E., Attia, A.S., Eldenshary Eel, D., 2003. Hypericum perforatum extract demonstrates antioxidant properties against elevated rat brain oxidative status induced by amnestic dose of scopolamine. Pharmacol Biochem Behav. 76**,** 525-33.

Ellman, G.L., Courtney, K.D., Andres, V., Jr., Feather-Stone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol. 7**,** 88-95.

Erecinska, M., Silver, I.A., 2001. Tissue oxygen tension and brain sensitivity to hypoxia. Respir Physiol. 128**,** 263-76.

Ferrer, I., 2009. Altered mitochondria, energy metabolism, voltage-dependent anion channel, and lipid rafts converge to exhaust neurons in Alzheimer's disease. J Bioenerg Biomembr. 41**,** 425-31.

Fiske, C.H., Subbarow, Y., 1927. The Nature of the "Inorganic Phosphate" in Voluntary Muscle. Science. 65**,** 401-3.

Frank, J., Kamal-Eldin, A., Lundh, T., Maatta, K., Torronen, R., Vessby, B., 2002. Effects of dietary anthocyanins on tocopherols and lipids in rats. J Agric Food Chem. 50**,** 7226-30.

Frussa-Filho, R., Barbosa-Junior, H., Silva, R.H., Da Cunha, C., Mello, C.F., 1999. Naltrexone potentiates the anxiolytic effects of chlordiazepoxide in rats exposed to novel environments. Psychopharmacology (Berl). 147**,** 168-73.

Fujisawa, H., Kajikawa, K., Ohi, Y., Hashimoto, Y., Yoshida, H., 1965. Movement of radioactive calcium in brain slices and influences on it of protoveratrine, ouabain, potassium chloride and cocaine. Jpn J Pharmacol. 15**,** 327-34.

Geering, K., 1997. Na,K-ATPase. Curr Opin Nephrol Hypertens. 6**,** 434-9.

Glushchenko, T.S., Izvarina, N.L., 1997. Na+,K(+)-ATPase activity in neurons and glial cells of the olfactory cortex of the rat brain during the

development of long-term potentiation. Neurosci Behav Physiol. 27**,** 49-52.

Grisar, T., Guillaume, D., Delgado-Escueta, A.V., 1992. Contribution of Na+,K(+)-ATPase to focal epilepsy: a brief review. Epilepsy Res. 12**,** 141-9.

Gron, G., Brandenburg, I., Wunderlich, A.P., Riepe, M.W., 2006. Inhibition of hippocampal function in mild cognitive impairment: targeting the cholinergic hypothesis. Neurobiol Aging. 27**,** 78-87.

Gutierres, J.M., Carvalho, F.B., Rosa, M.M., Schmatz, R., Rodrigues, M., Vieira, J.M., Mazzanti, C.M., Morsch, V.M., Rubin, M.A., Schetinger, M.R.C., Spanevello, R.M., 2012a. Protective effect of α-Tocopherol on memory deficits and Na+,K+-ATPase and acetylcholinesterase activities in rats with diet-induced hypercholesterolemia. Biomedicine & Aging Pathology. 2**,** 73-80.

Gutierres, J.M., Carvalho, F.B., Schetinger, M.R., Rodrigues, M.V., Schmatz, R., Pimentel, V.C., Vieira, J.M., Rosa, M.M., Marisco, P., Ribeiro, D.A., Leal, C., Rubin, M.A., Mazzanti, C.M., Spanevello, R., 2012b. Protective effects of anthocyanins on the ectonucleotidase activity in the impairment of memory induced by scopolamine in adult rats. Life Sci. 91**,** 1221-8.

Gutierres, J.M., Kaizer, R.R., Schmatz, R., Mazzanti, C.M., Vieira, J.M., Rodrigues, M.V., Jaques, J., Carvalho, F., Zanini, D., Morsch, V.M., Schetinger, M.R., Spanevello, R.M., 2012c. alpha-Tocopherol regulates ectonucleotidase activities in synaptosomes from rats fed a high-fat diet. Cell Biochem Funct. 30**,** 286-92.

Hattori, N., Kitagawa, K., Higashida, T., Yagyu, K., Shimohama, S., Wataya, T., Perry, G., Smith, M.A., Inagaki, C., 1998. CI-ATPase and Na+/K(+)-ATPase activities in Alzheimer's disease brains. Neurosci Lett. 254**,** 141-4.

Hauptmann, S., Scherping, I., Drose, S., Brandt, U., Schulz, K.L., Jendrach, M., Leuner, K., Eckert, A., Muller, W.E., 2009. Mitochondrial dysfunction: an early event in Alzheimer pathology accumulates with age in AD transgenic mice. Neurobiol Aging. 30**,** 1574-86.

Honer, W.G., Prohovnik, I., Smith, G., Lucas, L.R., 1988. Scopolamine reduces frontal cortex perfusion. J Cereb Blood Flow Metab. 8**,** 635-41.

Hut, R.A., Van der Zee, E.A., 2011. The cholinergic system, circadian rhythmicity, and time memory. Behav Brain Res. 221**,** 466-80.

Ibach, B., Haen, E., 2004. Acetylcholinesterase inhibition in Alzheimer's Disease. Curr Pharm Des. 10**,** 231-51.

Izquierdo, I., 1989. Mechanism of action of scopolamine as an amnestic. Trends Pharmacol Sci. 10**,** 175-7.

Jeong, E.J., Lee, K.Y., Kim, S.H., Sung, S.H., Kim, Y.C., 2008. Cognitive-enhancing and antioxidant activities of iridoid glycosides from Scrophularia buergeriana in scopolamine-treated mice. Eur J Pharmacol. 588**,** 78-84.

Jorgensen, P.L., Hakansson, K.O., Karlish, S.J., 2003. Structure and mechanism of Na,K-ATPase: functional sites and their interactions. Annu Rev Physiol. 65**,** 817-49.

Kahkonen, M.P., Hopia, A.I., Heinonen, M., 2001. Berry phenolics and their antioxidant activity. J Agric Food Chem. 49**,** 4076-82.

Kahkonen, M.P., Heinonen, M., 2003. Antioxidant activity of anthocyanins and their aglycons. J Agric Food Chem. 51**,** 628-33.

Kaplan, J.H., 2002. Biochemistry of Na,K-ATPase. Annu Rev Biochem. 71**,** 511-35.

Kasa, P., Rakonczay, Z., Gulya, K., 1997. The cholinergic system in Alzheimer's disease. Prog Neurobiol. 52**,** 511-35.

Kim, H.G., Ju, M.S., Shim, J.S., Kim, M.C., Lee, S.H., Huh, Y., Kim, S.Y., Oh, M.S., 2010. Mulberry fruit protects dopaminergic neurons in toxin-induced Parkinson's disease models. Br J Nutr. 104**,** 8-16.

Kirvela, O.A., Kanto, J.H., Raty, H.M., Iisalo, E., 1994. Anticholinergic drugs: effects on oxygen consumption and energy expenditure. Anesth Analg. 78**,** 995-9.

Klinkenberg, I., Blokland, A., 2010. The validity of scopolamine as a pharmacological model for cognitive impairment: a review of animal behavioral studies. Neurosci Biobehav Rev. 34**,** 1307-50.

Kodavanti, P.R., 1999. Measurement of calcium buffering by intracellular organelles in brain. Methods Mol Med. 22**,** 171-6.

Kopelman, M.D., Corn, T.H., 1988. Cholinergic 'blockade' as a model for cholinergic depletion. A comparison of the memory deficits with those of

Alzheimer-type dementia and the alcoholic Korsakoff syndrome. Brain. 111 (Pt 5), 1079-110.

Krikorian, R., Eliassen, J.C., Boespflug, E.L., Nash, T.A., Shidler, M.D., 2010a. Improved cognitive-cerebral function in older adults with chromium supplementation. Nutr Neurosci. 13, 116-22.

Krikorian, R., Shidler, M.D., Nash, T.A., Kalt, W., Vinqvist-Tymchuk, M.R., Shukitt-Hale, B., Joseph, J.A., 2010b. Blueberry supplementation improves memory in older adults. J Agric Food Chem. 58, 3996-4000.

Krikorian, R., Boespflug, E.L., Fleck, D.E., Stein, A.L., Wightman, J.D., Shidler, M.D., Sadat-Hossieny, S., 2012. Concord Grape Juice Supplementation and Neurocognitive Function in Human Aging. J Agric Food Chem.

Lane, R.M., Potkin, S.G., Enz, A., 2006. Targeting acetylcholinesterase and butyrylcholinesterase in dementia. Int J Neuropsychopharmacol. 9, 101-24.

Lees, G.J., Lehmann, A., Sandberg, M., Hamberger, A., 1990. The neurotoxicity of ouabain, a sodium-potassium ATPase inhibitor, in the rat hippocampus. Neurosci Lett. 120, 159-62.

Lingrel, J.B., Williams, M.T., Vorhees, C.V., Moseley, A.E., 2007. Na,K-ATPase and the role of alpha isoforms in behavior. J Bioenerg Biomembr. 39, 385-9.

Maggi, S., Noale, M., Zambon, A., Limongi, F., Romanato, G., Crepaldi, G., 2008. Validity of the ATP III diagnostic criteria for the metabolic syndrome in an elderly Italian Caucasian population: the Italian Longitudinal Study on Aging. Atherosclerosis. 197, 877-82.

Malatova, Z., Nicak, A., Marsala, J., 1980. Acetylcholinesterase activity and its molecular forms in selected regions of the dog's nervous system. Physiol Bohemoslov. 29, 415-22.

Manach, C., Scalbert, A., Morand, C., Remesy, C., Jimenez, L., 2004. Polyphenols: food sources and bioavailability. Am J Clin Nutr. 79, 727-47.

Mata, A.M., Sepulveda, M.R., 2010. Plasma membrane Ca-ATPases in the nervous system during development and ageing. World J Biol Chem. 1, 229-34.

Mata, A.M., Berrocal, M., Sepulveda, M.R., 2011. Impairment of the activity of the plasma membrane Ca(2)(+)-ATPase in Alzheimer's disease. Biochem Soc Trans. 39**,** 819-22.

Matsumoto, H., Inaba, H., Kishi, M., Tominaga, S., Hirayama, M., Tsuda, T., 2001. Orally administered delphinidin 3-rutinoside and cyanidin 3-rutinoside are directly absorbed in rats and humans and appear in the blood as the intact forms. J Agric Food Chem. 49**,** 1546-51.

Mazzanti, C.M., Spanevello, R.M., Pereira, L.B., Goncalves, J.F., Kaizer, R., Correa, M., Ahmed, M., Mazzanti, A., Festugatto, R., Graca, D.L., Morsch, V.M., Schetinger, M.R., 2006. Acetylcholinesterase activity in rats experimentally demyelinated with ethidium bromide and treated with interferon beta. Neurochem Res. 31**,** 1027-34.

McGhie, T.K., Ainge, G.D., Barnett, L.E., Cooney, J.M., Jensen, D.J., 2003. Anthocyanin glycosides from berry fruit are absorbed and excreted unmetabolized by both humans and rats. J Agric Food Chem. 51**,** 4539-48.

Mehta, P.D., 2007. Amyloid beta protein as a marker or risk factor of Alzheimer's disease. Curr Alzheimer Res. 4**,** 359-63.

Messer, W.S., Jr., Bohnett, M., Stibbe, J., 1990. Evidence for a preferential involvement of M1 muscarinic receptors in representational memory. Neurosci Lett. 116**,** 184-9.

Michaelis, E.K., Michaelis, M.L., Chang, H.H., Kitos, T.E., 1983. High affinity Ca2+-stimulated Mg2+-dependent ATPase in rat brain synaptosomes, synaptic membranes, and microsomes. J Biol Chem. 258**,** 6101-8.

Min, J., Yu, S.W., Baek, S.H., Nair, K.M., Bae, O.N., Bhatt, A., Kassab, M., Nair, M.G., Majid, A., 2011. Neuroprotective effect of cyanidin-3-O-glucoside anthocyanin in mice with focal cerebral ischemia. Neurosci Lett. 500**,** 157-61.

Miranda, K.M., Espey, M.G., Wink, D.A., 2001. A rapid, simple spectrophotometric method for simultaneous detection of nitrate and nitrite. Nitric Oxide. 5**,** 62-71.

Miyazawa, T., Nakagawa, K., Kudo, M., Muraishi, K., Someya, K., 1999. Direct intestinal absorption of red fruit anthocyanins, cyanidin-3-glucoside and cyanidin-3,5-diglucoside, into rats and humans. J Agric Food Chem. 47**,** 1083-91.

Moseley, A.E., Williams, M.T., Schaefer, T.L., Bohanan, C.S., Neumann, J.C., Behbehani, M.M., Vorhees, C.V., Lingrel, J.B., 2007. Deficiency in Na,K-ATPase alpha isoform genes alters spatial learning, motor activity, and anxiety in mice. J Neurosci. 27**,** 616-26.

Mudnic, I., Budimir, D., Modun, D., Gunjaca, G., Generalic, I., Skroza, D., Katalinic, V., Ljubenkov, I., Boban, M., 2011. Antioxidant and Vasodilatory Effects of Blackberry and Grape Wines. J Med Food.

Musial, A., Bajda, M., Malawska, B., 2007. Recent developments in cholinesterases inhibitors for Alzheimer's disease treatment. Curr Med Chem. 14**,** 2654-79.

Nagy, A., Delgado-Escueta, A.V., 1984. Rapid preparation of synaptosomes from mammalian brain using nontoxic isoosmotic gradient material (Percoll). J Neurochem. 43**,** 1114-23.

Nishizuka, T., Fujita, Y., Sato, Y., Nakano, A., Kakino, A., Ohshima, S., Kanda, T., Yoshimoto, R., Sawamura, T., 2011. Procyanidins are potent inhibitors of LOX-1: a new player in the French Paradox. Proc Jpn Acad Ser B Phys Biol Sci. 87**,** 104-13.

Paleari, L., Grozio, A., Cesario, A., Russo, P., 2008. The cholinergic system and cancer. Semin Cancer Biol. 18**,** 211-7.

Parsons, J.T., Sun, D.A., DeLorenzo, R.J., Churn, S.B., 2004. Neuronal-specific endoplasmic reticulum Mg(2+)/Ca(2+) ATPase Ca(2+) sequestration in mixed primary hippocampal culture homogenates. Anal Biochem. 330**,** 130-9.

Passamonti, S., Vrhovsek, U., Vanzo, A., Mattivi, F., 2005. Fast access of some grape pigments to the brain. J Agric Food Chem. 53**,** 7029-34.

Pavlov, K.V., Sokolov, V.S., 2000. Electrogenic ion transport by Na+,K+-ATPase. Membr Cell Biol. 13**,** 745-88.

Ramirez, M.R., Izquierdo, I., do Carmo Bassols Raseira, M., Zuanazzi, J.A., Barros, D., Henriques, A.T., 2005. Effect of lyophilised Vaccinium berries on memory, anxiety and locomotion in adult rats. Pharmacol Res. 52**,** 457-62.

Rang Oh, S., Jin Kim, S., Hyun Kim, D., Hoon Ryu, J., Ahn, E.M., Wook Jung, J., 2012. Angelica keiskei ameliorates scopolamine-induced memory impairments in mice. Biol Pharm Bull.

Ray, C.A., Blin, J., Chase, T.N., Piercey, M.F., 1992. Effects of cholinergic agonists on regional brain energy metabolism in the scopolamine-treated rat. Neuropharmacology. 31**,** 1193-9.

Rohn, T.T., Hinds, T.R., Vincenzi, F.F., 1993. Ion transport ATPases as targets for free radical damage. Protection by an aminosteroid of the Ca2+ pump ATPase and Na+/K+ pump ATPase of human red blood cell membranes. Biochem Pharmacol. 46**,** 525-34.

Rose, A.M., Valdes, R., Jr., 1994. Understanding the sodium pump and its relevance to disease. Clin Chem. 40**,** 1674-85.

Rubin, M.A., Albach, C.A., Berlese, D.B., Bonacorso, H.G., Bittencourt, S.R., Queiroz, C.M., Maixner, A.E., Mello, C.F., 2000a. Anxiolytic-like effects of 4-phenyl-2-trichloromethyl-3H-1, 5-benzodiazepine hydrogen sulfate in mice. Braz J Med Biol Res. 33**,** 1069-73.

Rubin, M.A., Boemo, R.L., Jurach, A., Rojas, D.B., Zanolla, G.R., Obregon, A.D., Souza, D.O., Mello, C.F., 2000b. Intrahippocampal spermidine administration improves inhibitory avoidance performance in rats. Behav Pharmacol. 11**,** 57-61.

Saija, A., Princi, P., D'Amico, N., De Pasquale, R., Costa, G., 1990. Effect of Vaccinium myrtillus anthocyanins on triiodothyronine transport into brain in the rat. Pharmacol Res. 22 Suppl 3**,** 59-60.

Sakurai, T., Kato, T., Mori, K., Takano, E., Watabe, S., Nabeshima, T., 1998. Nefiracetam elevates extracellular acetylcholine level in the frontal cortex of rats with cerebral cholinergic dysfunctions: an in vivo microdialysis study. Neurosci Lett. 246**,** 69-72.

Sambamurti, K., Greig, N.H., Lahiri, D.K., 2002. Advances in the cellular and molecular biology of the beta-amyloid protein in Alzheimer's disease. Neuromolecular Med. 1**,** 1-31.

Sansoni, P., Vescovini, R., Fagnoni, F., Biasini, C., Zanni, F., Zanlari, L., Telera, A., Lucchini, G., Passeri, G., Monti, D., Franceschi, C., Passeri, M., 2008. The immune system in extreme longevity. Exp Gerontol. 43**,** 61-5.

Scalbert, A., Williamson, G., 2000. Dietary intake and bioavailability of polyphenols. J Nutr. 130**,** 2073S-85S.

Scarpini, E., Cogiamanian, F., 2003. Alzheimer's disease: from molecular pathogenesis to innovative therapies. Expert Rev Neurother. 3**,** 619-30.

Scarpini, E., Scheltens, P., Feldman, H., 2003. Treatment of Alzheimer's disease: current status and new perspectives. Lancet Neurol. 2**,** 539-47.

Scuri, R., Lombardo, P., Cataldo, E., Ristori, C., Brunelli, M., 2007. Inhibition of Na+/K+ ATPase potentiates synaptic transmission in tactile sensory neurons of the leech. Eur J Neurosci. 25**,** 159-67.

Shen, Z.X., 2004. Brain cholinesterases: I. The clinico-histopathological and biochemical basis of Alzheimer's disease. Med Hypotheses. 63**,** 285-97.

Shih, P.H., Chan, Y.C., Liao, J.W., Wang, M.F., Yen, G.C., 2010. Antioxidant and cognitive promotion effects of anthocyanin-rich mulberry (Morus atropurpurea L.) on senescence-accelerated mice and prevention of Alzheimer's disease. J Nutr Biochem. 21**,** 598-605.

Skou, J.C., Esmann, M., 1992. The Na,K-ATPase. J Bioenerg Biomembr. 24**,** 249-61.

Southam, E., Thomas, P.K., King, R.H., Goss-Sampson, M.A., Muller, D.P., 1991. Experimental vitamin E deficiency in rats. Morphological and functional evidence of abnormal axonal transport secondary to free radical damage. Brain. 114 (Pt 2)**,** 915-36.

Spencer, J.P., 2010. The impact of fruit flavonoids on memory and cognition. Br J Nutr. 104 Suppl 3**,** S40-7.

Stone, W.S., Walser, B., Gold, S.D., Gold, P.E., 1991. Scopolamine- and morphine-induced impairments of spontaneous alternation performance in mice: reversal with glucose and with cholinergic and adrenergic agonists. Behav Neurosci. 105**,** 264-71.

Talavera, S., Felgines, C., Texier, O., Besson, C., Gil-Izquierdo, A., Lamaison, J.L., Remesy, C., 2005. Anthocyanin metabolism in rats and their distribution to digestive area, kidney, and brain. J Agric Food Chem. 53**,** 3902-8.

Therien, A.G., Blostein, R., 2000. Mechanisms of sodium pump regulation. Am J Physiol Cell Physiol. 279**,** C541-66.

Trevisan, G., Maldaner, G., Velloso, N.A., Sant'Anna Gda, S., Ilha, V., Velho Gewehr Cde, C., Rubin, M.A., Morel, A.F., Ferreira, J., 2009. Antinociceptive effects of 14-membered cyclopeptide alkaloids. J Nat Prod. 72**,** 608-12.

Varadinova, M.G., Docheva-Drenska, D.I., Boyadjieva, N.I., 2009. Effects of anthocyanins on learning and memory of ovariectomized rats. Menopause. 16**,** 345-9.

Veitch, N.C., Grayer, R.J., 2008. Flavonoids and their glycosides, including anthocyanins. Nat Prod Rep. 25**,** 555-611.

Verkhratsky, A., Rodriguez, J.J., Parpura, V., 2012. Calcium signalling in astroglia. Mol Cell Endocrinol. 353**,** 45-56.

Wang, L.S., Stoner, G.D., 2008. Anthocyanins and their role in cancer prevention. Cancer Lett. 269**,** 281-90.

Wesnes, K.A., Simpson, P.M., White, L., Pinker, S., Jertz, G., Murphy, M., Siegfried, K., 1991. Cholinesterase inhibition in the scopolamine model of dementia. Ann N Y Acad Sci. 640**,** 268-71.

Williams, C.A., Grayer, R.J., 2004. Anthocyanins and other flavonoids. Nat Prod Rep. 21**,** 539-73.

Xiao, A.Y., Wei, L., Xia, S., Rothman, S., Yu, S.P., 2002. Ionic mechanism of ouabain-induced concurrent apoptosis and necrosis in individual cultured cortical neurons. J Neurosci. 22**,** 1350-62.

Yamaguchi, M., 2012. Role of regucalcin in brain calcium signaling: involvement in aging. Integr Biol (Camb). 4**,** 825-37.

Yoshida, K., Mori, M., Kondo, T., 2009. Blue flower color development by anthocyanins: from chemical structure to cell physiology. Nat Prod Rep. 26**,** 884-915.

Youdim, K.A., Dobbie, M.S., Kuhnle, G., Proteggente, A.R., Abbott, N.J., Rice-Evans, C., 2003. Interaction between flavonoids and the blood-brain barrier: in vitro studies. J Neurochem. 85**,** 180-92.

Zaidi, A., 2010. Plasma membrane Ca-ATPases: Targets of oxidative stress in brain aging and neurodegeneration. World J Biol Chem. 1**,** 271-80.

Zakut, H., Matzkel, A., Schejter, E., Avni, A., Soreq, H., 1985. Polymorphism of acetylcholinesterase in discrete regions of the developing human fetal brain. J Neurochem. 45**,** 382-9.

Legends

Scheme 1. *Exposure design*

Table 1 - Effect of anthocyanins treatment (200 mg kg^{-1}) and scopolamine injection (1 mg kg^{-1}) on memory parameters in adult rats.

Table 2 - Effect of scopolamine and anthocyanin on the behavior of rats (number of crossing and rearing responses) and on foot shock sensitivity (flinch, jump and vocalization).

Figure 1 - Effect of anthocyanins (200 mg kg^{-1}) and scopolamine (1 mg kg^{-1}) on anxiety-like behavior in adult rats. Bars represent the mean ± SEM. * Represents a significant saline or ANT versus saline or SCO interaction (Two way ANOVA).

Figure 2 - Effect of anthocyanins (200 mg kg^{-1}) and scopolamine (1 mg kg^{-1}) on AChE activity in synaptosomes (A) and supernatant (B) in cerebral cortex and hippocampus of rats. Bars represent the mean ± SEM. * Represents a significant saline or ANT versus saline or SCO interaction (Two way ANOVA)

Figure 3 - Effect of anthocyanins (200 mg kg^{-1}) and scopolamine (1 mg kg^{-1}) on Na^{+}, K^{+}-ATPase (A) and Ca^{2+}-ATPase (B) activities in cerebral cortex and hippocampus of adult rats. Bars represent the mean ± SEM. * Represents a significant saline or ANT versus saline or SCO interaction (Two way ANOVA)

Figure 4 - Effect of anthocyanins (200 mg kg^{-1}) and scopolamine (1 mg kg^{-1}) on NOx levels in cerebral cortex and hippocampus of rats. Bars represent the mean ± SEM (Two way ANOVA).

Table 1. Effect of anthocyanin treatment (200 mg kg^{-1}) and scopolamine injection (1 mg kg^{-1}) on memory parameters in adult rats.

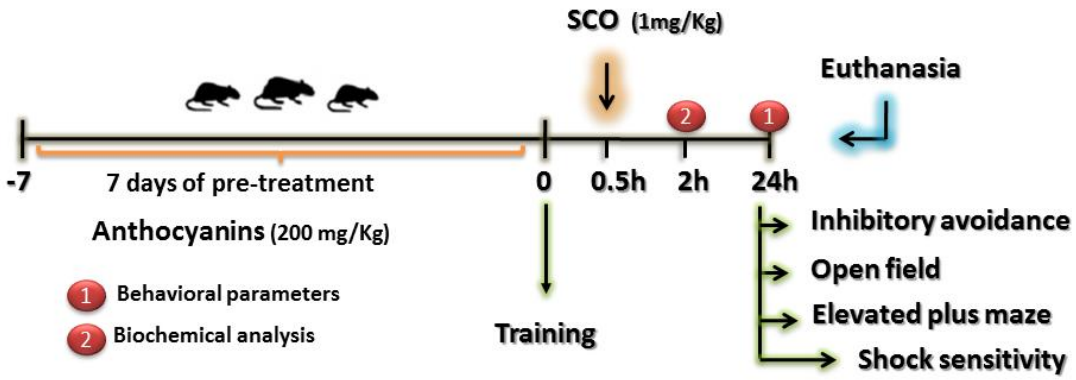
	Latency of Training		***Latency of tes***	
Groups	*Mean* ± SEM	*minimum*	*median*	*maximum*
Control	7.50 ± 1.99	69.00	175.00	300.00
ANT	8.37 ± 1.79	110.00	210.00	300.00
SCO	5.30 ± 1.59	25.00	66.50 *	110.00
SCO+ ANT	8.22 ± 1.35	116.00	218.00 #	300.00
Statistical Analysis	$F_{(3.36)}$= 0.77; P>0.05	--	H=9.75; P<0.01	--

Data training are means ± SEM. Data Test are the median ± interquartile, 8-10 animals in each group. * *P<0.05* compared with the others groups. # *P<0.05* compared with SCO group by the Dunn's nonparametric multiple comparisons task (Scheirer-Ray-Hare extension of two way ANOVA, nonparametric test).

Table 2 - Effect of scopolamine and anthocyanin on the open field and on foot shock sensitivity test.

	Open Field		*Foot shock sensitivity*		
Groups	***Crossing***	***Rearing***	***Flinch (mA)***	***Jump (mA)***	***Vocalization (mA)***
Control	21.75 ± 3.13	16.00 ± 2.28	0.36 ± 0.01	0.45 ± 0.02	0.35 ± 0.05
ANT	17.25 ± 2.19	13.63 ± 2.09	0.41 ± 0.03	0.36 ± 0.02	0.41 ± 0.03
SCO	22.10 ± 2.57	18.00 ± 2.96	0.34 ± 0.01	0.43 ± 0.02	0.44 ± 0.02
SCO+ ANT	23.89 ± 3.01	20.22 ± 2.36	0.37 ± 0.03	0.33 ± 0.02	0.41 ± 0.03
Statistical Analysis	$F_{(3.36)}$= 0.99; *p>0.05*	$F_{(3.36)}$= 0.13; *P>0.05*	$F_{(3.36)}$= 1.30; *P>0.05*	$F_{(3.36)}$= 0.48; *P>0.05*	$F_{(3.36)}$= 1.11; *P>0.05*

Data are means ± SEM for 8-10 animals in each group.



Scheme 1

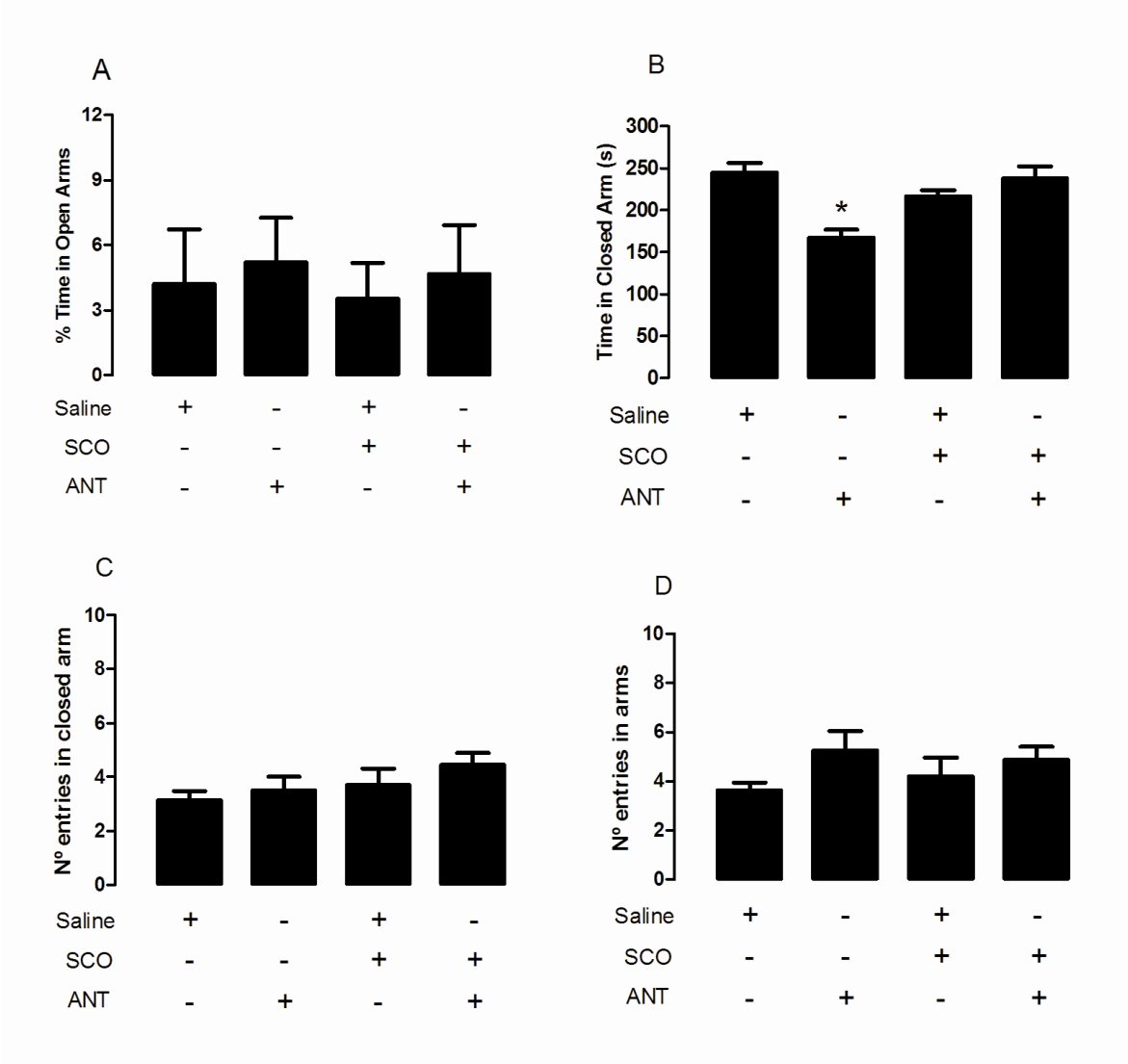

A
% Time in Open Arms
12
9
6
3
0
Saline + - + -
SCO - - + +
ANT - + - +
B
Time in Closed Arm (s)
300
250
200
150
100
50
0
*
Saline + - + -
SCO - - + +
ANT - + - +
C
Nº entries in closed arm
10
8
6
4
2
0
Saline + - + -
SCO - - + +
ANT - + - +
D
Nº entries in arms
10
8
6
4
2
0
Saline + - + -
SCO - - + +
ANT - + - +

Figure 1

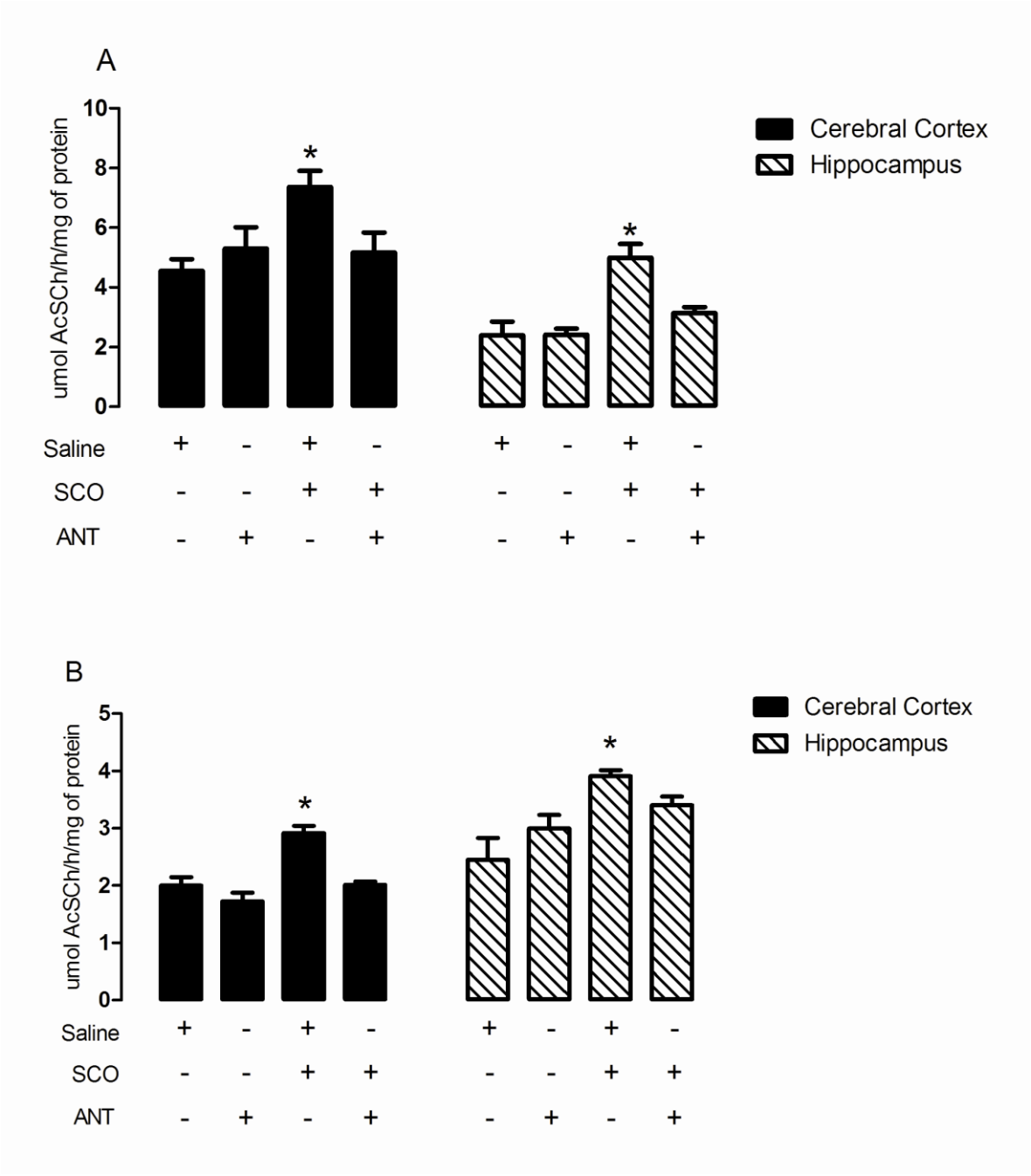

A
umol AcSCh/h/mg of protein
10
8
6
4
2
0
*
*
Cerebral Cortex
Hippocampus
Saline + - + - + - + -
SCO - - + + - - + +
ANT - + - + - + - +
B
umol AcSCh/h/mg of protein
5
4
3
2
1
0
*
*
Cerebral Cortex
Hippocampus
Saline + - + - + - + -
SCO - - + + - - + +
ANT - + - + - + - +

Figure 2

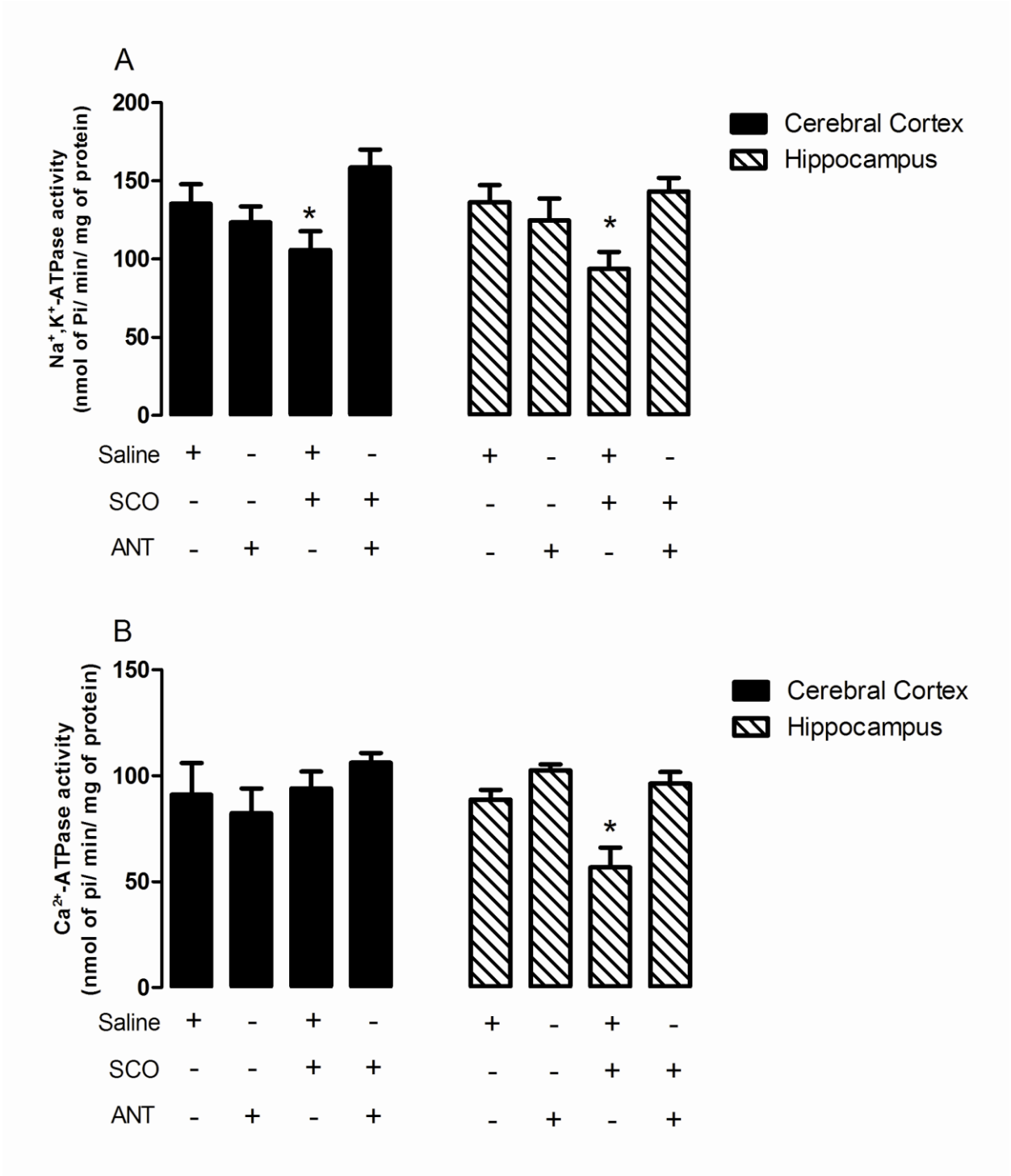

A
Na+,K+-ATPase activity
(nmol of Pi/ min/ mg of protein)
200
150
100
50
0
*
*
Cerebral Cortex
Hippocampus
Saline + - + - + - + -
SCO - - + + - - + +
ANT - + - + - + - +
B
Ca2+-ATPase activity
(nmol of pi/ min/ mg of protein)
150
100
50
0
*
Cerebral Cortex
Hippocampus
Saline + - + - + - + -
SCO - - + + - - + +
ANT - + - + - + - +

Figure 3

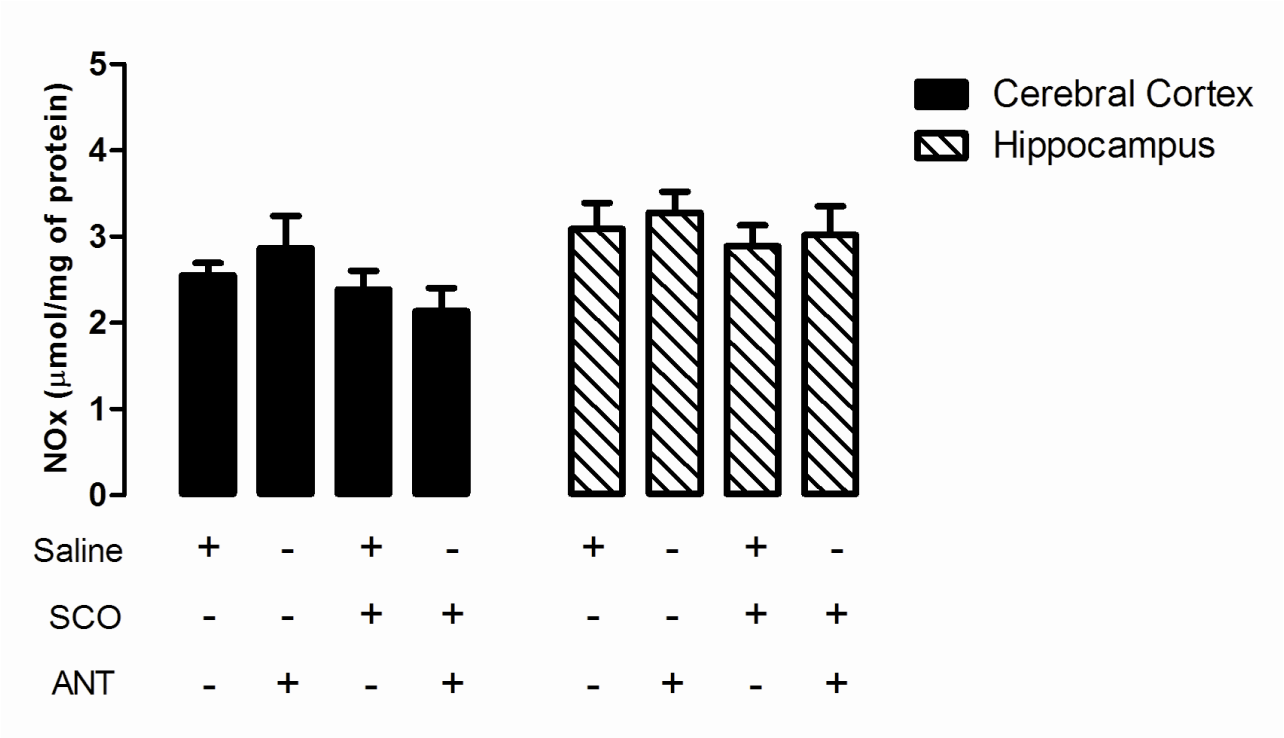

NOx (µmol/mg of protein)
5
4
3
2
1
0
Cerebral Cortex
Hippocampus
Saline + - + - + - + -
SCO - - + + - - + +
ANT - + - + - + - +

Figure 4

4. Artigo I

4.1. Protective effects of anthocyanins on the ectonucleotidase activity in the impairment of memory induced by scopolamine in adult rats

Contents lists available at SciVerse ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Protective effects of anthocyanins on the ectonucleotidase activity in the impairment of memory induced by scopolamine in adult rats

Jessié M. Gutierres [a,*], Fabiano B. Carvalho [a], Maria R.C. Schetinger [a], Marília V. Rodrigues [a], Roberta Schmatz [a], Victor C. Pimentel [a], Juliano M. Vieira [a], Michele M. Rosa [a], Patrícia Marisco [a], Daniela A. Ribeiro [a], Claudio Leal [a], Maribel A. Rubin [a], Cinthia M. Mazzanti [c], Roselia Spanevello [b,**]

[a] Departamento de Química, Universidade Federal de Santa Maria, Santa Maria, RS 97105-900, Brazil
[b] Centro de Ciências Químicas, Farmacêuticas e de Alimentos, Universidade Federal de Pelotas, Campus Universitário-Capão do Leão 96010-900 Pelotas, RS, Brazil
[c] Clínica de Pequenos animais, Centro de Ciências Rurais, Universidade Federal de Santa Maria, Santa Maria, RS 97105-900, Brazil

ARTICLE INFO

Article history:
Received 24 March 2012
Accepted 19 September 2012

Keywords:
Anthocyanins
Scopolamine
Ectonucleotidases
Memory

ABSTRACT

Aims: We investigated whether the treatment with anthocyanins prevents the scopolamine-induced memory deficits and whether ectonucleotidase activities and purine levels are altered in the cerebral cortex (CC) and hippocampus (HC) in this model of mnemonic deficit in rats.
Main methods: The animals were divided into 4 experimental groups: control (vehicle), anthocyanins (Antho), scopolamine (SCO), and scopolamine plus anthocyanins (SCO+Antho). After seven days of treatment, they were tested in the inhibitory avoidance task and open field test and submitted to euthanasia. The CC and the HC were collected for biochemical assays. The effect of treatment with Antho (200 mg kg^{-1}, i.p.) was investigated in rats trained to a stable level of performance and post-treated with SCO (1 mg kg^{-1}, i.p. 30 min after training).
Key findings: The treatment with SCO decreased the step-down latency in inhibitory avoidance task. Antho prevented the scopolamine-induced memory impairment and also the increase of NTPDase activity in the CC and HC. Furthermore, the treatment with anthocyanins prevents the decrease in 5′-nucleotidase activity and the increase in adenosine deaminase activity induced by SCO in HC. In addition, the treatment with Antho prevented the decrease in ATP levels induced by SCO in the CC and HC.
Significance: Our results show that scopolamine may affect purinergic enzymatic cascade or cause alterations in energy metabolism inducing loss of memory. In contrast Antho could reverse these changes, suggesting a neuroprotective effect of Antho on ectonucleotidase activities and neuronal energetic metabolism.

Introduction

The extracellular nucleotide ATP and its nucleoside derivative adenosine are important signaling molecules involved in innumerous physiological and pathological functions (Bours et al., 2006; Burnstock, 2006a). It has been shown that ATP and adenosine have an array of functions in the central nervous system (CNS) acting as neurotransmitters and activating purinergic and adenosinergic receptors, respectively (Burnstock, 2011; Zimmermann, 1996, 1999, 2006). The levels of extracellular ATP and adenosine may be related to processes of learning and memory formation, since various evidences point to LTP and LTD and synaptic plasticity as a neural basis for cognitive processes (Cooke and Bliss, 2006; Howland and Wang, 2008; Lovinger, 2010; Malenka, 1994).

The extracellular levels of ATP and adenosine are regulated by a cascade of cell-surface-bound enzymes named ectonucleotidases (Battastini et al., 1991). The NTPDase is an enzyme that hydrolyzes ATP and ADP into AMP, which is subsequently converted to adenosine by the enzyme 5′-nucleotidase (Battastini et al., 1991; Zimmermann, 1996, 1999, 2006). Moreover, adenosine is cleaved by the enzyme adenosine deaminase in inosine in the synaptic cleft (Robson et al., 2006, 2005). Together, these enzymes constitute an organized enzymatic cascade for the regulation of nucleotide-mediated signaling, controlling rate, degradation, and nucleoside formation (Abbracchio et al., 2009; Burnstock, 2009, 2011; Schetinger et al., 2007, 2001). The involvement of ectonucleotidases on the process of learning and memory in rats has also been described (Bonan et al., 1998, 2000; Pereira et al., 2002).

Scopolamine (SCO) is a non-selective muscarinic cholinergic antagonist which produces a transient memory impairment in rodents (Klinkenberg and Blokland, 2010). It has been also reported that SCO reduces frontal cortex perfusion in young humans (Honer et al., 1988) and impairs the energetic metabolism reducing the ATP levels in the cerebral cortex of rats (Blin et al., 1994; Ray et al., 1992). The impairment of mitochondrial function and reduction of ATP levels

* Correspondence to: J.M. Gutierres, Laboratório de Enzimologia Toxicológica, Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria, Santa Maria, RS 97105-900, Brazil. Tel./fax: +55 55 3220 9557.
** Corresponding author. Tel./fax: +55 55 3220 9557.
E-mail addresses: jessiegutierres@hotmail.com (J.M. Gutierres), rspanevello@gmail.com (R. Spanevello).

0024-3205/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.lfs.2012.09.013

are pathological conditions found in neurodegenerative diseases such as Alzheimer's disease (AD), which is closely linked to the decline of cognitive processes (Ferrer, 2009; Hauptmann et al., 2009).

Anthocyanins belong to the flavonoid family; they present phenolic groups in their chemical structure and give colors to flowers and fruits of a great variety of plants (Veitch and Grayer, 2008; Williams and Grayer, 2004; Yoshida et al., 2009). It has been shown that anthocyanins are potent antioxidants (Kahkonen and Heinonen, 2003; Kahkonen et al., 2001) and have neuroprotective properties (Del Rio et al., 2010), preventing neurotoxicity induced by reperfusion damage model of cerebral ischemia (Min et al., 2011; Shin et al., 2006), by deleterious effects found in models of Parkinson's (Kim et al., 2010) and AD (Shih et al., 2010). Altogether these evidences suggest that flavonoid compounds also have beneficial effects on memory and cognition (Spencer, 2010). In fact, it has been described that anthocyanins improve memory in old rats in Morris water maze (Andres-Lacueva et al., 2005) and also cognition in older humans (Krikorian et al., 2010).

In this context, we sought to investigate if anthocyanin treatment prevented the scopolamine-induced memory deficits and if the ecto-nucleotidase enzymes as well as the ATP and adenosine levels are involved.

Materials and methods

Chemicals

Nucleotides, Trizma Base, Percoll, and Coomassie Brilliant Blue G were purchased from Sigma Chemical Co (St. Luis, MO, USA). Scopolamine HCl (SCO) was purchased from Sigma-Aldrich Chemie GmbH (Schnelldorf, Germany), and anthocyanins were extracted and purified from grape skin and gently donated by Christian Hansen A/S. All other reagents used in the experiments were of analytical grade and of the highest purity.

Animals

This study used three months old adult male Wistar rats (350–400 g). They were kept in the Central Animal House of Federal University of Santa Maria in colony cages at an ambient temperature of 25 ± 2 °C and relative humidity of 45–55% with 12 h light/dark cycles. They had free access to standard rodent pelleted diet and water ad libitum. Experiments were conducted in accordance with the Institutional Ethical Committee of the Federal University of Santa Maria.

Exposure

Seven to ten animals per group were usually tested in the experiments (Scheme 1). Rats were treated i.p. with Antho (200 mg kg^{-1} body weight) daily for 7 days (around 10 a.m.). The doses were calculated as free base form. Scopolamine was dissolved in saline and injected i.p. at a dose of 1 mg kg^{-1}, and administered 30 min after the training in inhibitory avoidance apparatus in accordance with that previously described (Ali and Arafa, 2011). The dose of anthocyanins was chosen on the basis of previous studies indicating neuroprotection (Manach et al., 2004; Saija et al., 1990; Varadinova et al., 2009). In addition, the daily intake of anthocyanins in the residents of the United States is estimated to be about 200 mg or about 9-fold higher than that of other dietary flavonoids, and this also served as a basis for this study (Manach et al., 2004; Wang and Stoner, 2008).

Behavioral procedure

Inhibitory avoidance task

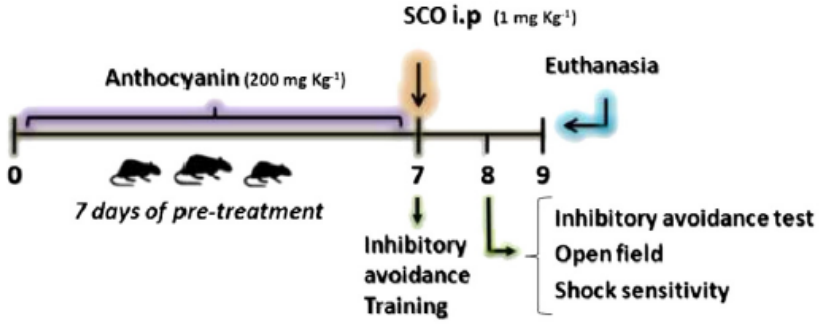
In the final day of treatment with anthocyanins (7th day), the animals were subjected to training in a step-down inhibitory avoidance apparatus in accordance with that previously described (Rubin et al., 2000), and then the animals received SCO (i.p. 1 mg kg^{-1}) 30 min after training. Next, 24 h after the training the animals were subjected to test in a step-down inhibitory avoidance task. Briefly, the rats were subjected to a single 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 rat was 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.

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., 2000). 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



Scheme 1. Exposure design.

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 halothane atmosphere before being killed by decapitation and brain were removed and separated into cerebral cortex (CC) and hippocampus (HC) and placed in a solution of Medium I (320 mM sucrose, 0.1 mM EDTA and 5 mM HEPES, pH 7.5) on ice (Gutierres et al., 2012). The brain structures were gently homogenized in a glass potter in Medium I. Protein was determined by the Coomassie Blue method with that previously described (Bradford, 1976), using bovine serum albumin as standard solution.

Synaptosome preparation

Synaptosomes were isolated essentially as that previously described (Nagy and Delgado-Escueta, 1984), using a discontinuous Percoll gradient. The CC and HC 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 isoosmotic 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 isoosmotic 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 isoosmotic 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 for NTPDase and 5′-nucleotidase assays.

Assay of lactate dehydrogenase

The integrity of the synaptosomes preparations was confirmed by determining 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, Brasil).

Assay of NTPDase and 5′-nucleotidase activities

The NTPDase enzymatic assay of the synaptosomes was carried out in plates with a reaction medium containing 5 mM KCl, 1.5 mM $CaCl_2$, 0.1 mM EDTA, 10 mM glucose, 225 mM sucrose and 45 mM Tris–HCl buffer, pH 8.0, in a final volume of 200 µl as described in a previous work from our laboratory (Schetinger et al., 2000). The 5′-nucleotidase activity was determined with that previously described (Heymann et al., 1984) in a reaction medium containing 10 mM $MgSO_4$ and 100 mM Tris–HCl buffer, pH 7.5, in a final volume of 200 µl. In synaptosomes 20 µl of enzyme preparation (8–12 µg of protein) was added to the reaction mixture and pre-incubated at 37 °C for 10 min. The reaction was initiated by the addition of ATP or ADP to obtain a final concentration of 1.0 mM and incubation proceeded for 20 min. For AMP hydrolysis, the 5′-nucleotidase activity was carried out as previously described and the final concentration of the nucleotide AMP added was 2 mM. The reactions were stopped by the addition of 200 µl of 10% trichloroacetic acid (TCA) to provide a final concentration of 5%. Subsequently, the tubes were chilled on ice for 10 min. Released inorganic phosphate (Pi) was assayed using malachite green as the colorimetric reagent and KH_2PO_4 as standard (Chan et al., 1986). All samples were run in triplicate. Enzyme specific activities are reported as nmol Pi released/min/mg of protein. Adenosine deaminase activity was estimated spectrophotometrically by the method of Giusti (1974), and the values were expressed as U/L of protein for ADA.

Analysis of purine levels in cerebral cortex and hippocampus by high pressure liquid chromatography

Sample preparation

ATP and its breakdown products were extracted with that previously described (Ryder, 1985). Briefly, different amounts of the CC or HC were weighted and homogenized with 0.6 M perchloric acid at 0 °C for 1 min with an Ultra-turrax homogenizer (model T 18, IKA® Works Inc., Wilmington, Del., USA). The homogenate was centrifuged at 2000×*g* for 10 min, and the supernatant was immediately neutralized to pH 6.5 to 6.8 with 1 M potassium hydroxide.

High performance liquid chromatography analysis

High performance liquid chromatography (HPLC) was performed with a Shimadzu (Kyoto, Japan) equipment composed of a reciprocating pump model LC-20AT, a degasser model DGU-20A5, a diode array detector (DAD) model SPD-M20A, auto-sampler (SIL-20A) and integrator model CBM-20A, operated by the LC Solution 1.22 SP1 software. Separation was achieved with a Phenomenex Synergi 4 µm Fusion RP-80A column (150×4.60 mm, 4 µm) with precolumn, using 0.04 M potassium dihydrogen orthophosphate (KH_2PO_4) and 0.06 M dipotassium hydrogen orthophosphate (K_2HPO_4) as mobile phase A and acetonitrile as mobile phase B. A gradient elution was used according to the specifications previously described (Scherer et al., 2005), at a flow rate of 0.7 ml/min. Mobile phases were filtered through a 0.45 µm Millipore filter prior to analysis, and all the reagents utilized were of HPLC grade. Purines in the samples (ATP, ADP, AMP and adenosine) were identified by their retention times and DAD spectrum (in the range 200–400 nm), and quantified by comparison of the peak's area with standards. The results are expressed by pmol of the different compounds per ml of sample.

Protein determination

Protein was measured by the Coomassie Blue method with that previously described (Bradford, 1976), using bovine serum albumin as standard.

Statistical analysis

Statistical analysis of latency test was carried by Scheirer–Ray–Hare (extension of the Kruskal–Wallis test or two-way ANOVA). Foot shock sensitivity test was analyzed by unpaired *t* test. Enzymatic activity, nucleotide and nucleoside levels, crossing and rearing responses were analyzed by one- or two-way ANOVA, followed by Tukey's multiple range tests. $P<0.05$ was considered to represent a significant difference in all experiments.

Results

Behavioral tests

Anthocyanins prevent the impairment of memory induced by scopolamine

Fig. 1 shows the effect of the treatment with anthocyanins (Antho) and scopolamine-induced (SCO) memory deficits, on step-down latencies. Statistical analysis of testing (*nonparametric two-way ANOVA*) showed a significant scopolamine (1 mg kg^{-1}) vs anthocyanins (200 mg kg^{-1}) or vehicle interaction (Control), revealing that treatment with anthocyanins reverses the impairment of memory induced by SCO [H = 9.75; *P* 0.01]. Statistical analysis of training (*one-way ANOVA*) showed no difference between groups [F(3,31) = 0.77;

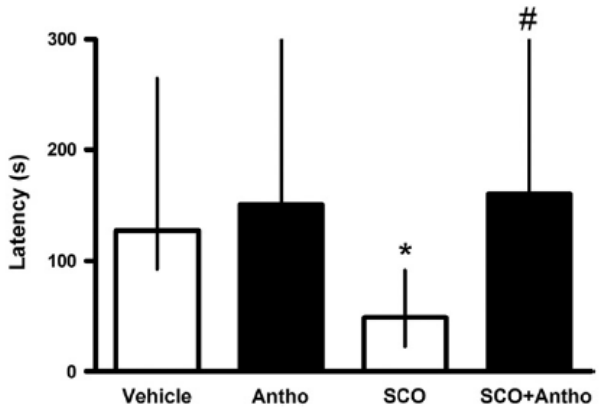


Fig. 1. Oral administration of anthocyanins (200 mg kg^{-1}) once a day during 7 days reverses the impairment of memory induced by scopolamine (1 mg kg^{-1}) in adult rats. Data are the median ± interquartile range for 8–10 animals in each group. *$P<0.05$ compared with the other groups, #$P<0.05$ compared with the scopolamine (SCO) group by the Scheirer–Ray–Hare nonparametric multiple comparisons task.

P 0.05]. Because motivational disparities in the training session may account for differences in inhibitory avoidance at testing, experiments were performed to assess whether SCO or Antho affected shock threshold, or locomotor ability of the animals. Statistical analysis of open-field data (*one-way ANOVA*) revealed that scopolamine did not alter the number of crossing [$F(3,31)=0.99$; $P<0.05$] or rearing [$F(3,31)=0.13$; $P<0.05$] responses in a subsequent open-field test session, suggesting that neither SCO nor Antho caused gross motor disabilities at testing (Table 1). Moreover, SCO did not alter foot shock sensitivity, as demonstrated by the similar flinch [unpaired t test: $F(3,31)=1.30$; $P>0.05$], jump [unpaired t test: $F(3,31)=4.48$; $P>0.05$] and vocalization [unpaired t test: $F(3,31)=1.11$; $P>0.05$] thresholds exhibited by the animals. These data suggest that neither treatment with Antho administered before nor SCO administered after training of inhibitory avoidance caused motor disabilities or altered foot shock sensitivity (Table 1).

Enzymatic activities

Anthocyanins prevent the increase in NTPDase activity induced by scopolamine

Fig. 2 shows the effect of Antho and SCO on the activity of NTPDase in the CC and HC of rats. SCO increased the NTPDase activity in the CC (Fig. 2A) and HC (Fig. 2B) using ATP nucleotide as substrate. Antho prevented the increase in NTPDase activity induced by SCO in the CC [$F(1,20)=7.371$; P 0.05, Fig. 2A] and HC [$F(1,20)=6.397$; P 0.05, Fig. 2B]. SCO also increased the NTPDase activity in HC using ADP nucleotide as substrate and Antho prevents this effect [$F(1,20)=6.397$; P 0.05, Fig. 2D]. No significant differences in the activity of NTPDase in the CC were observed in the groups [$F(1,20)=0.002$; P 0.05, Fig. 2C].

Anthocyanins prevent the decrease in 5′-nucleotidase activity induced by scopolamine

Fig. 3 shows the effect of Antho and SCO on the activity of 5′-nucleotidase in the CC (Fig. 3A) and HC (Fig. 3B) of rats. Scopolamine decreased the 5′-nucleotidase activity in the HC. Antho prevented the decrease in 5′-nucleotidase activity induced by SCO in the HC [$F(1,20)=12.98$; P 0.01, Fig. 3B]. No significant differences in the activity of 5′-Nucleotidase in the CC was observed [$F(1,20)=0.288$; P 0.05, Fig. 3A].

Anthocyanins prevent the increase in adenosine deaminase activity induced by scopolamine

Fig. 4 shows the effect of Antho and SCO on the activity of adenosine deaminase in the CC (Fig. 4A) and HC (Fig. 4B) of rats. SCO increased the adenosine deaminase activity in the HC. Antho prevented the increase in adenosine deaminase activity induced by SCO in the HC [$F(1,20)=4.897$, P 0.05, Fig. 4B]. No significant differences in the activity of adenosine deaminase in the CC was observed [$F(1,20)=0.071$, P 0.05, Fig. 4A].

Content of nucleotides and nucleosides of adenine

Anthocyanins can restore decrease in purine levels induced by scopolamine

Table 2 shows the effect of Antho and SCO on the nucleotide levels in the CC of rats. SCO decreased the ATP levels CC in relation to the all groups. However, when the animals received anthocyanins (SCO + Antho), the levels of ATP were similar to those of the vehicle group [$F(1,12)=14.23$; P 0.01]. No significant differences in ADP [$F(1,12)=0.036$; P 0.05], AMP [$F(1,12)=3.002$; P 0.05] and adenosine levels were observed [$F(1,12)=4.013$; P 0.05].

Table 3 shows the effect of Antho and SCO on the nucleotides levels in the HC of rats. SCO decreased the ATP levels in the HC in relation to all the groups. However, when the animals received anthocyanins (SCO + Antho), the levels of ATP were similar to those of the vehicle group [$F(1,12)=5.648$; P 0.05]. No significant differences in ADP [$F(1,12)=3.593$; P 0.05], AMP [$F(1,12)=1.684$; P 0.05] and adenosine [$F(1,12)=0.373$; P 0.05] levels were observed.

Discussion

In the present study, we have evaluated the potential preventive role of the anthocyanins (Antho) in the scopolamine-induced memory deficits in rats. Our results have shown that scopolamine impaired the memory consolidation in rats trained in the inhibitory avoidance task. Interestingly, in this study we found that anthocyanins at a dose of 200 mg kg^{-1} during 7 days did not improve the memory of rats in the inhibitory avoidance task, but it prevented the memory deficits induced by scopolamine (SCO) (Scheme 1).

SCO is an alkaloid derived from *Atropa belladonna* (Zhang et al., 2008) and acts as a competitive antagonist of the muscarinic acetylcholine receptor (mAChR) (Wang et al., 2003). For this reason, SCO is used to compromise cholinergic neurotransmission and, in some

Table 1
Effect of anthocyanins (200 mg kg^{-1}) and scopolamine (1 mg kg^{-1}) on the latency of training, and behavior of rats (number of crossing and rearing responses) in the open-field immediately after the inhibitory avoidance testing session and on foot shock sensitivity (flinch, jump and vocalization).

Group	Training	Crossing	Rearing	Flinch (mA)	Jump (mA)	Vocalization (mA)
Vehicle	7.50 ± 1.99	21.75 ± 3.13	16.00 ± 2.28	0.36 ± 0.01	0.45 ± 0.02	0.35 ± 0.05
Antho	8.37 ± 1.79	17.25 ± 2.19	13.63 ± 2.09	0.41 ± 0.03	0.36 ± 0.02	0.41 ± 0.03
SCO	5.30 ± 1.59	22.10 ± 2.57	18.00 ± 2.96	0.34 ± 0.01	0.43 ± 0.02	0.44 ± 0.02
SCO + Antho	8.22 ± 1.35	23.89 ± 3.01	20.22 ± 2.36	0.37 ± 0.03	0.33 ± 0.02	0.41 ± 0.03
Statistical analysis	$F(3,31)=0.77$; $P>0.05$	$F(3,31)=0.99$; $P>0.05$	$F(3,31)=1.30$; $P>0.05$	$F(3,31)=1.30$; $P>0.05$	$F(3,31)=4.48$; $P>0.05$	$F(3,31)=1.11$; $P>0.05$

Data are means + SEM for 6–10 animals in each group.

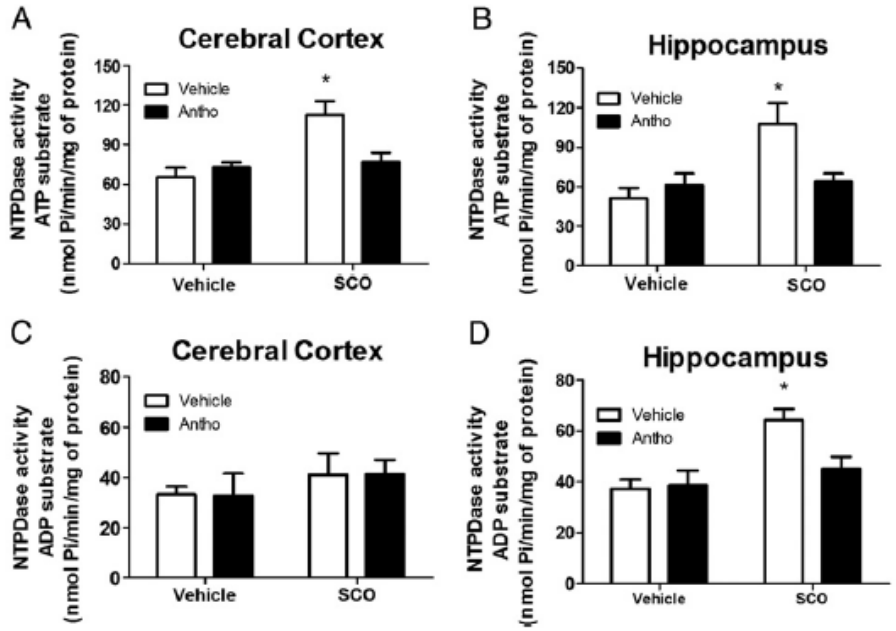


Fig. 2. Effects of Antho and SCO on NTPDase activity in synaptosomes of the cerebral cortex and hippocampus using ATP (A and B) and ADP (C and D) respectively as substrates. Bars represent the mean ± SEM. *Represents a significant difference in all groups, $P<0.05$. (ANOVA *two-way*, Tukey test).

ways, mimics the memory deficit observed in diseases characterized by impairment in cholinergic neurotransmission, such as AD (Christensen et al., 1992; Kopelman and Corn, 1988; Wesnes et al., 1991).

It has been reported that dietary supplementation of antioxidant-rich berries (e.g., blueberry, strawberry) can improve the learning and memory in the aged animal (Williams and Grayer, 2004; Williams et al., 2008). The daily intake of anthocyanin chosen for this study (200 mg kg^{-1}) is in agreement with the consumption of that in the residents of the United States population (Manach et al., 2004; Wang and Stoner, 2008). Moreover, previous studies indicate that a daily intake of 200 mg/kg of anthocyanins has protective effects in rats, mice and humans (Bao et al., 2008a, 2008b; Choi et al., 2007; Heinonen, 2001) and improve learning and memory in female rats ovariectomized (Varadinova et al., 2009). Furthermore, there are few studies using acute treatment with anthocyanins, because diverse number of flavonoid compounds, including anthocyanins, seem to require a short-term or long-term to accumulate and promote beneficial effects in the brain of rodents (Barros et al., 2006; Hassimotto and Lajolo, 2011; Ke et al., 2011; Min et al., 2011; Yang et al., 2011). Acute doses of anthocyanins may result in immediate beneficial changes on the peripheral homeostasis and biochemical tissue parameters (Matsumoto et al., 2001; Rossi et al., 2003). However, no previous study of acute and subacute doses of anthocyanins associated with learning and memory have been observed at this point.

Additionally, studies have shown that Antho have the ability to improve 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). Interestingly, it was also shown to be a cognitive improvement in older humans (Krikorian et al., 2010). In line with this view, studies with the Cyanidin-3-O-glucopyranoside (Cy-3G), an isolated Antho and abundant in colorful vegetables and fruits, has recently been identified as potent neuroprotective phytochemical since this compound protects against Aβ peptide-mediated cytotoxicity in SH-SY5Y neurocytes (Tarozzi et al., 2008, 2007, 2010) and also can reduce cerebral ischemia damage and age-related neuronal deficits (Shin et al., 2006).

Despite SCO's classical use as an amnesic agent, there are a lot of discrepancies in relation to SCO effects in locomotion. Some studies, in fact, challenge the viability of SCO use as a cognitive impairer, questioning if the alterations in behavior are related to peripheral locomotor effects, instead of memory disruption (Klinkenberg and Blokland, 2010). As shown in Table 1, there were no changes in none of the parameters analyzed. We have also shown in a pilot test (data not shown) that

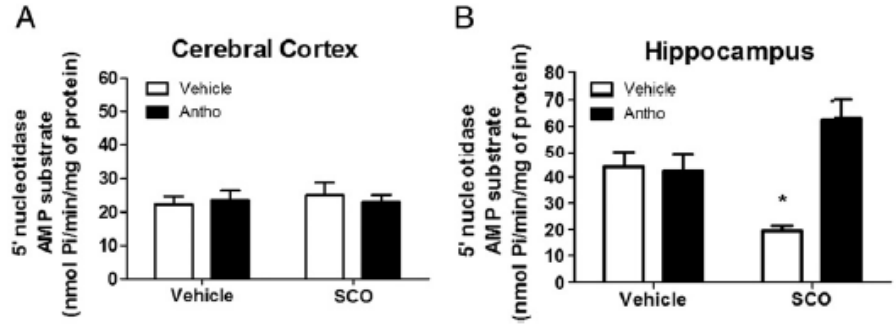


Fig. 3. Effects of Antho and SCO on 5'-nucleotidase activity in synaptosomes of the cerebral cortex and hippocampus using AMP as substrate. Bars represent the mean ± SEM. *Represents a significant difference in all groups, $P<0.05$. (ANOVA *two-way*, Tukey test).

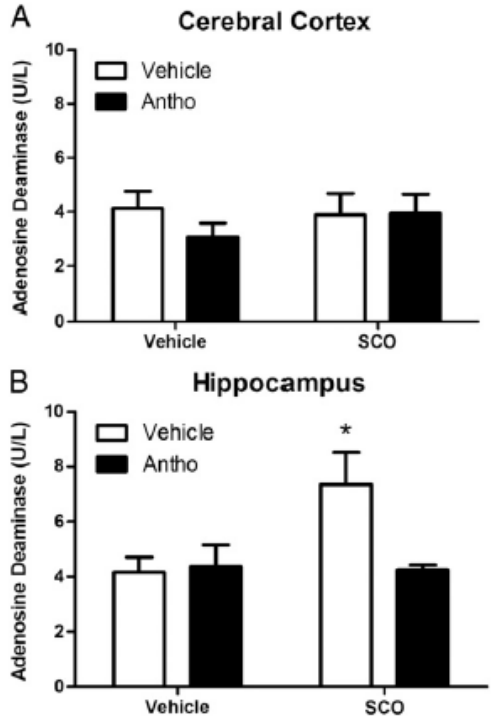


Fig. 4. Effects of Antho and SCO on adenosine deaminase activity in synaptosomes of the cerebral cortex and hippocampus using adenosine as substrate. Bars represent the mean ± SEM. *Represents a significant difference in all groups, $P<0.05$. (ANOVA *two-way*, Tukey test).

exposure to SCO for 1 h immediately before inhibitory avoidance training did not impact training performance, since control and scopolamine-treated animals' training session latencies did not change.

In the brain, information storage is involved with an increase in efficiency of synaptic stimulatory pathways (Wieraszko, 1996; Wieraszko and Ehrlich, 1994). Since the first demonstration of ATP-dependent release of CNS stimulation (Holton, 1959), there is a growing interest in the role of ATP and adenosine in synaptic transmission (Cunha and Ribeiro, 2000). ATP and adenosine act as neurotransmitters modulating purinergic and adenosinergic receptors, respectively (Zimmermann, 1996, 1999, 2006). It has been reported that ATP and adenosine modulate low term potentiation (LTP) and low term depression (LTD) in neurons (de Mendonca et al., 2002; Fujii, 2004; Wieraszko and Ehrlich, 1994; Yamazaki et al., 2003) and also contribute to neuronal synaptic plasticity (Costenla et al., 1999; de Mendonca and Ribeiro, 1997; Wieraszko, 1996). These processes are related with learning and memory formation (Cooke and Bliss, 2006; Howland and Wang, 2008; Lovinger, 2010; Malenka, 1994).

In CNS, the NTPDase is an important enzyme involved in the purinergic neurotransmission. Our results show that an increase in the NTPDase activity in synaptosomes of the CC and HC in rats treated with SCO leads to a reduction of extracellular ATP in the synaptic cleft, which may impair the purinergic signaling since it reduces the availability of extracellular ATP. In addition, these enzymes differ in their ratios and preferably for the substrate hydrolysis. The NTPDase 1 hydrolyzes ATP and ADP in the same way; also, NTPDases 2, 3 and 8 hydrolyze ATP more than ADP, and NTPDase 4 preferentially hydrolyzes UDP (Zimmermann, 2001). Based on these findings, we believe that the effects found in the hippocampus and cerebral cortex can be related to the activity of different enzyme isoforms. Enzymatic hydrolysis of ATP and ADP in the hippocampus suggests the involvement of NTPDase 1, whereas in the cerebral cortex other isoforms with greater affinity to ATP can be involved (Fig. 2). Furthermore, the reduction on the ATP levels can impair processes like the establishment of the neuronal LTP (Fujii, 2004; Min et al., 2011; Wieraszko and Ehrlich, 1994; Yamazaki et al., 2003) affecting memory formation (Cooke and Bliss, 2006; Howland and Wang, 2008; Lovinger, 2010; Malenka, 1994). These results corroborate our findings when compared with the memory evaluated in the inhibitory avoidance task, where the amnesic effect induced by SCO was prevented by the treatment with Antho.

The next step was to assess the 5′-nucleotidase and adenosine deaminase activity. As it can be seen in Fig. 3, SCO decreased the activity of 5′-nucleotidase in synaptosomes of the HC (Fig. 3B), and the treatment with Antho was able to prevent this decrease. Furthermore, our results showed that 5′-nucleotidase and ADA activities seem to be more sensitive in the HC than CC, so we believe that ectonucleotidase in the hippocampus is more affected by scopolamine (see Figs. 3 and 4). However, we can not exclude that specific regions of the cerebral cortex which are known to participate in memory formation, as the posterior cingulate and entorhinal cortex (Lima et al., 2009; Pereira et al., 2005, 2001; Souza et al., 2002), are also affected by SCO administration, so ectonucleotidase activities were measured in the whole cerebral cortex and not in specific regions, might have been the reason that no significant differences were observed in this brain structure. The activity of this enzyme is very important because this leads to production of extracellular adenosine (Burnstock, 2006a, b; Robson et al., 2006; Schetinger et al., 2001). It was also observed that SCO increased the activity of adenosine deaminase, and this effect was prevented by the treatment with Antho. The 5′-nuleotidase and adenosine deaminase are key-enzymes in the regulation of extracellular levels of adenosine in the synaptic cleft. A decrease of 5′-nucleotidase activity reduces the adenosine formation and the increase of adenosine deaminase activity increases the hydrolysis of adenosine to inosine. Thus, the effect of SCO on these enzymes leads to an increased removal of extracellular adenosine decreasing its levels, which may lead to impairment of the adenosinergic neurotransmission.

The depletion of extracellular adenosine can disrupt memory formation since adenosine has been reported as an important neuromodulator in the establishment of LTP and LTD, as well as in synaptic plasticity (de Mendonca et al., 2002), (Costenla et al., 1999; de Mendonca and Ribeiro, 1997; Wieraszko, 1996). Based on the activity of the ectonucleotidases, it can be seen that the treatment with SCO increased the hydrolysis of

Table 2
Effect of anthocyanins (200 mg kg^{-1}) and scopolamine (1 mg kg^{-1}) on the nucleotides levels (ATP, ADP, AMP and Adenosine) in the cerebral cortex of rats. The data were analyzed by *two-way* ANOVA followed by Tukey's test and expressed in nmol of nucleotide/mg of tissue. *Represents a significant difference in all groups, $P<0.05$.

Groups	ATP	ADP	AMP	Adenosine
Vehicle	0.78 ± 0.089	0.56 ± 0.187	0.40 ± 0.017	0.91 ± 0.128
Antho	0.83 ± 0.130	0.71 ± 0.040	0.54 ± 0.089	1.14 ± 0.258
SCO	0.58 ± 0.032*	0.59 ± 0.089	0.54 ± 0.057	1.17 ± 0.174
SCO + Antho	0.90 ± 0.023	0.77 ± 0.052	0.45 ± 0.161	1.07 ± 0.104
Statistical analysis	F(1,12) = 14.23; $P<0.01$	F(1,12) = 0.036; $P>0.05$	F(1,12) = 3.002; $P>0.05$	F(1,12) = 4.013; $P>0.05$

Data are means ± SEM for 4 animals in each group (cerebral cortex).

Table 3
Effect of anthocyanins (200 mg kg^{-1}) and scopolamine (1 mg kg^{-1}) on the nucleotides levels (ATP, ADP, AMP and Adenosine) in the hippocampus of rats. The data were analyzed by *two-way* ANOVA followed by Tukey's test and expressed in nmol of nucleotide/mg of tissue. *Represents a significant difference in all groups, $P<0.05$.

Groups	ATP	ADP	AMP	Adenosine
Vehicle	1.0 ± 0.044	0.39 ± 0.089	0.22 ± 0.090	0.46 ± 0.087
Antho	0.93 ± 0.030	0.45 ± 0.064	0.28 ± 0.080	0.54 ± 0.110
SCO	0.44 ± 0.023*	0.46 ± 0.059	0.30 ± 0.100	0.59 ± 0.094
SCO + Antho	0.89 ± 0.043	0.41 ± 0.056	0.26 ± 0.089	0.51 ± 0.098
Statistical analysis	F(1,12) = 5.648; $P<0.05$	F(1,12) = 3.593; $P>0.05$	F(1,12) = 1.648; $P>0.05$	F(1,12) = 0.375; $P>0.05$

Data are means ± SEM for 4 animals in each group (hippocampus).

Hauptmann S, Scherping I, Drose S, Brandt U, Schulz KL, Jendrach M, et al. Mitochondrial dysfunction: an early event in Alzheimer pathology accumulates with age in AD transgenic mice. Neurobiol Aging 2009;30:1574–86.

Havsteen BH. The biochemistry and medical significance of the flavonoids. Pharmacol Ther 2002;96:67-202.

Heinonen M. Anthocyanins as dietary antioxidants. Third international conference on natural antioxidants and anticarcinogens in food, health and disease (NADH); 2001. p. 6–9.

Heymann D, Reddington M, Kreutzberg GW. Subcellular localization of 5′-nucleotidase in rat brain. J Neurochem 1984;43:971–8.

Holton P. The liberation of adenosine triphosphate on antidromic stimulation of sensory nerves. J Physiol 1959;145:494–504.

Honer WG, Prohovnik I, Smith G, Lucas LR. Scopolamine reduces frontal cortex perfusion. J Cereb Blood Flow Metab 1988;8:635–41.

Howland JG, Wang YT. Synaptic plasticity in learning and memory: stress effects in the hippocampus. Prog Brain Res 2008;169:145–58.

Kahkonen MP, Heinonen M. Antioxidant activity of anthocyanins and their aglycons. J Agric Food Chem 2003;51:628–33.

Kahkonen MP, Hopia AI, Heinonen M. Berry phenolics and their antioxidant activity. J Agric Food Chem 2001;49:4076–82.

Ke Z, Liu Y, Wang X, Fan Z, Chen G, Xu M, et al. Cyanidin-3-glucoside ameliorates ethanol neurotoxicity in the developing brain. J Neurosci Res 2011;89:1676–84.

Kim HG, Ju MS, Shim JS, Kim MC, Lee SH, Huh Y, et al. Mulberry fruit protects dopaminergic neurons in toxin-induced Parkinson's disease models. Br J Nutr 2010;104:8-16.

Kirvela OA, Kanto JH, Raty HM, Iisalo E. Anticholinergic drugs: effects on oxygen consumption and energy expenditure. Anesth Analg 1994;78:995–9.

Klinkenberg I, Blokland A. The validity of scopolamine as a pharmacological model for cognitive impairment: a review of animal behavioral studies. Neurosci Biobehav Rev 2010;34:1307–50.

Kopelman MD, Corn TH. Cholinergic 'blockade' as a model for cholinergic depletion. A comparison of the memory deficits with those of Alzheimer-type dementia and the alcoholic Korsakoff syndrome. Brain 1988;111(Pt 5):1079–110.

Krikorian R, Shidler MD, Nash TA, Kalt W, Vinqvist-Tymchuk MR, Shukitt-Hale B, et al. Blueberry supplementation improves memory in older adults. J Agric Food Chem 2010;58:3996–4000.

Lima RH, Rossato JI, Furini CR, Bevilaqua LR, Izquierdo I, Cammarota M. Infusion of protein synthesis inhibitors in the entorhinal cortex blocks consolidation but not reconsolidation of object recognition memory. Neurobiol Learn Mem 2009;91:466–72.

Lovinger DM. Neurotransmitter roles in synaptic modulation, plasticity and learning in the dorsal striatum. Neuropharmacology 2010;58:951–61.

Malenka RC. Synaptic plasticity in the hippocampus: LTP and LTD. Cell 1994;78:535–8.

Manach C, Scalbert A, Morand C, Remesy C, Jimenez L. Polyphenols: food sources and bioavailability. Am J Clin Nutr 2004;79:727–47.

Matsumoto H, Inaba H, Kishi M, Tominaga S, Hirayama M, Tsuda T. Orally administered delphinidin 3-rutinoside and cyanidin 3-rutinoside are directly absorbed in rats and humans and appear in the blood as the intact forms. J Agric Food Chem 2001;49:1546–51.

Min J, Yu SW, Baek SH, Nair KM, Bae ON, Bhatt A, et al. Neuroprotective effect of cyanidin-3-O-glucoside anthocyanin in mice with focal cerebral ischemia. Neurosci Lett 2011;500:157–61.

Mudnic I, Budimir D, Modun D, Gunjaca G, Generalic I, Skroza D, et al. Antioxidant and vasodilatory effects of blackberry and grape wines. J Med Food 2012;15(3):315–21.

Nagy A, Delgado-Escueta AV. Rapid preparation of synaptosomes from mammalian brain using nontoxic isoosmotic gradient material (Percoll). J Neurochem 1984;43:1114–23.

Pereira P, Ardenghi P, Mello e Souza T, Medina JH, Izquierdo I. Training in the step-down inhibitory avoidance task time-dependently increases cAMP-dependent protein kinase activity in the entorhinal cortex. Behav Pharmacol 2001;12:217–20.

Pereira GS, Mello e Souza T, Battastini AM, Izquierdo I, Sarkis JJ, Bonan CD. Effects of inhibitory avoidance training and/or isolated foot-shock on ectonucleotidase activities in synaptosomes of the anterior and posterior cingulate cortex and the medial precentral area of adult rats. Behav Brain Res 2002;128:121–7.

Pereira GS, Rossato JI, Sarkis JJ, Cammarota M, Bonan CD, Izquierdo I. Activation of adenosine receptors in the posterior cingulate cortex impairs memory retrieval in the rat. Neurobiol Learn Mem 2005;83:217–23.

Ray CA, Blin J, Chase TN, Piercey MF. Effects of cholinergic agonists on regional brain energy metabolism in the scopolamine-treated rat. Neuropharmacology 1992;31:1193–9.

Robson SC, Wu Y, Sun X, Knosalla C, Dwyer K, Enjyoji K. Ectonucleotidases of CD39 family modulate vascular inflammation and thrombosis in transplantation. Semin Thromb Hemost 2005;31:217–33.

Robson SC, Sevigny J, Zimmermann H. The E-NTPDase family of ectonucleotidases: structure function relationships and pathophysiological significance. Purinergic Signal 2006;2:409–30.

Rossi A, Serraino I, Dugo P, Di Paola R, Mondello L, Genovese T, et al. Protective effects of anthocyanins from blackberry in a rat model of acute lung inflammation. Free Radic Res 2003;37:891–900.

Rubin MA, Boemo RL, Jurach A, Rojas DB, Zanolla GR, Obregon AD, et al. Intrahippocampal spermidine administration improves inhibitory avoidance performance in rats. Behav Pharmacol 2000;11:57–61.

Ryder JM. Determination of adenosine triphosphate and its breakdown products in fish muscle by high-performance liquid chromatography. J Agric Food Chem 1985;33:678–80.

Saija A, Princi P, D'Amico N, De Pasquale R, Costa G. Effect of *Vaccinium myrtillus* anthocyanins on triiodothyronine transport into brain in the rat. Pharmacol Res 1990;22(Suppl. 3):59–60.

Scherer R, Augusti PR, Steffens C, Bochi VC, Heckttheuer LH, Lazzari R, et al. Effect of slaughter method on postmortem changes of grass carp (*Ctenopharyngodon idella*) stored in icesti. J Food Sci 2005;70:348–53.

Schetinger MR, Porto NM, Moretto MB, Morsch VM, da Rocha JB, Vieira V, et al. New benzodiazepines alter acetylcholinesterase and ATPDase activities. Neurochem Res 2000;25:949–55.

Schetinger MR, Vieira VL, Morsch VM, Balz D. ATP and ADP hydrolysis in fish, chicken and rat synaptosomes. Comp Biochem Physiol B Biochem Mol Biol 2001;128:731–41.

Schetinger MR, Morsch VM, Bonan CD, Wyse AT. NTPDase and 5′-nucleotidase activities in physiological and disease conditions: new perspectives for human health. Biofactors 2007;31:77–98.

Shih PH, Chan YC, Liao JW, Wang MF, Yen GC. Antioxidant and cognitive promotion effects of anthocyanin-rich mulberry (*Morus atropurpurea* L.) on senescence-accelerated mice and prevention of Alzheimer's disease. J Nutr Biochem 2010;21:598–605.

Shin WH, Park SJ, Kim EJ. Protective effect of anthocyanins in middle cerebral artery occlusion and reperfusion model of cerebral ischemia in rats. Life Sci 2006;79:130–7.

Singh M, Arseneault M, Sanderson T, Murthy V, Ramassamy C. Challenges for research on polyphenols from foods in Alzheimer's disease: bioavailability, metabolism, and cellular and molecular mechanisms. J Agric Food Chem 2008;56:4855–73.

Souza MM, Mello e Souza T, Vinade ER, Rodrigues C, Choi HK, Dedavid e Silva TL, et al. Effects of posttraining treatments in the posterior cingulate cortex on short- and long-term memory for inhibitory avoidance in rats. Neurobiol Learn Mem 2002;77:202–10.

Spencer JP. The impact of fruit flavonoids on memory and cognition. Br J Nutr 2010;104(Suppl. 3):S40–7.

Stone WS, Walser B, Gold SD, Gold PE. Scopolamine- and morphine-induced impairments of spontaneous alternation performance in mice: reversal with glucose and with cholinergic and adrenergic agonists. Behav Neurosci 1991;105:264–71.

Tarozzi A, Morroni F, Hrelia S, Angeloni C, Marchesi A, Cantelli-Forti G, et al. Neuroprotective effects of anthocyanins and their in vivo metabolites in SH-SY5Y cells. Neurosci Lett 2007;424:36–40.

Tarozzi A, Merlicco A, Morroni F, Franco F, Cantelli-Forti G, Teti G, et al. Cyanidin 3-O-glucopyranoside protects and rescues SH-SY5Y cells against amyloid-beta peptide-induced toxicity. Neuroreport 2008;19:1483–6.

Tarozzi A, Morroni F, Merlicco A, Bolondi C, Teti G, Falconi M, et al. Neuroprotective effects of cyanidin 3-O-glucopyranoside on amyloid beta (25–35) oligomer-induced toxicity. Neurosci Lett 2010;473:72–6.

Vafeiadou K, Vauzour D, Spencer JP. Neuroinflammation and its modulation by flavonoids. Endocr Metab Immune Disord Drug Targets 2007;7:211–24.

Varadinova MG, Docheva-Drenska DI, Boyadjieva NI. Effects of anthocyanins on learning and memory of ovariectomized rats. Menopause 2009;16:345–9.

Veitch NC, Grayer RJ. Flavonoids and their glycosides, including anthocyanins. Nat Prod Rep 2008;25:555–611.

Wallerath T, Li H, Godtel-Ambrust U, Schwarz PM, Forstermann U. A blend of polyphenolic compounds explains the stimulatory effect of red wine on human endothelial NO synthase. Nitric Oxide 2005;12:97-104.

Wang LS, Stoner GD. Anthocyanins and their role in cancer prevention. Cancer Lett 2008;269:281–90.

Wang H, Lu Y, Chen HZ. Differentiating effects of anisodamine on cognitive amelioration and peripheral muscarinic side effects induced by pilocarpine in mice. Neurosci Lett 2003;344:173–6.

Ward CP, Redd K, Williams BM, Caler JR, Luo Y, McCoy JG. Ginkgo biloba extract: cognitive enhancer or antistress buffer. Pharmacol Biochem Behav 2002;72:913–22.

Wesnes KA, Simpson PM, White L, Pinker S, Jertz G, Murphy M, et al. Cholinesterase inhibition in the scopolamine model of dementia. Ann N Y Acad Sci 1991;640:268–71.

Wieraszko A. Extracellular ATP, as a neurotransmitter: its role in synaptic plasticity in the hippocampus. Acta Neurobiol Exp (Wars) 1996;56:637–48.

Wieraszko A, Ehrlich YH. On the role of extracellular ATP in the induction of long-term potentiation in the hippocampus. J Neurochem 1994;63:1731–8.

Williams CA, Grayer RJ. Anthocyanins and other flavonoids. Nat Prod Rep 2004;21:539–73.

Williams CM, El Mohsen MA, Vauzour D, Rendeiro C, Butler LT, Ellis JA, et al. Blueberry-induced changes in spatial working memory correlate with changes in hippocampal CREB phosphorylation and brain-derived neurotrophic factor (BDNF) levels. Free Radic Biol Med 2008;45:295–305.

Yamazaki Y, Kaneko K, Fujii S, Kato H, Ito K. Long-term potentiation and long-term depression induced by local application of ATP to hippocampal CA1 neurons of the guinea pig. Hippocampus 2003;13:81–92.

Yang H, Pang W, Lu H, Cheng D, Yan X, Cheng Y, et al. Comparison of metabolic profiling of cyanidin-3-O-galactoside and extracts from blueberry in aged mice. J Agric Food Chem 2011;59:2069–76.

Yoshida K, Mori M, Kondo T. Blue flower color development by anthocyanins: from chemical structure to cell physiology. Nat Prod Rep 2009;26:884–915.

Youdim KA, Dobbie MS, Kuhnle G, Proteggente AR, Abbott NJ, Rice-Evans C. Interaction between flavonoids and the blood–brain barrier: in vitro studies. J Neurochem 2003;85:180–92.

Zhang WW, Song MK, Cui YY, Wang H, Zhu L, Niu YY, et al. Differential neuropsychopharmacological influences of naturally occurring tropane alkaloids anisodamine versus scopolamine. Neurosci Lett 2008;443:241–5.

Zimmermann H. Biochemistry, localization and functional roles of ecto-nucleotidases in the nervous system. Prog Neurobiol 1996;49:589–618.

Zimmermann H. Two novel families of ectonucleotidases: molecular structures, catalytic properties and a search for function. Trends Pharmacol Sci 1999;20:231–6.

Zimmermann H. Ectonucleotidases: some recent developments and a note on nomenclature. Drug Dev Res 2001;52:44–56.

Zimmermann H. Nucleotide signaling in nervous system development. Pflugers Arch 2006;452:573–88.

5. Manuscrito II

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

Jessié M. Gutierres[1*], Fabiano B. Carvalho[1], Maria Rosa C. Schetinger[1], Patrícia Marisco[1], Paula Agostinho[4], Marília Rodrigues[1], Maribel A. Rubin[1], Roberta Schmatz[1], Cassia R. da Silva[1], Julia C. Farias[1], Giana de P. Cognato[2], Michele M. Rosa[1], Cinthia M. Mazzanti[1], Mauricio Bogo[2], Carla D. Bonan[2], Roselia Spanevello[3*]

[1] Programa de Pós-Graduação em Bioquímica Toxicológica, Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria, Av. Roraima, 97105-900 - Santa Maria, RS, Brazil.

[2] Laboratório de Neuroquímica e Psicofarmacologia, Faculdade de Biociências, Pontifícia Universidade Católica do Rio Grande do Sul. Avenida Ipiranga, 6681, 90619-900 Porto Alegre, RS, Brazil.

[3] Centro de Ciências Químicas, Farmacêuticas e de Alimentos, Universidade Federal de Pelotas, Campus Universitário- Capão do Leão 96010-900 Pelotas, RS, Brazil.

[4] Center for Neuroscience and Cell Biology, Faculty of Medicine, University of Coimbra, Coimbra, Portugal.

[*]Corresponding author:

Spanevello R. (Roselia Spanevello) and Gutierres J.M. (Jessié Martins Gutierres)

Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria, Av. Roraima, 97105-900 - Santa Maria, RS, Brasil.

E-mail address: rsspanevello@gmail.com and jessiegutierres@hotmail.com

Phone: +55 55 32209557

Abstract

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

Keywords: Anxiety-like behavior; Nitric oxide production, Acetylcholinesterase, Anthocyanin; Dementia; Memory.

1. Introduction

Anthocyanins belong to the flavonoid family, which present phenolic groups in their chemical structure and give colors to a great variety of flowers and fruits (Williams e Grayer, 2004; Yoshida, Mori *et al.*, 2009; Veitch e Grayer, 2011). It has been shown that anthocyanins (ANT) are potent antioxidants, and prove to be effective scavengers of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Kahkonen, Hopia *et al.*, 2001; Kahkonen e Heinonen, 2003), having a clear neuroprotective role (Del Rio, Borges *et al.*, 2010). ANT were shown to prevent neurotoxicity induced by: i) ethanol in developing brain mice (Ke, Liu *et al.*, 2011), ii) reperfusion damage model of cerebral ischemia (Shin, Park *et al.*, 2006; Min, Yu *et al.*, 2011), iii) deleterious effects found in models of Parkinson's (Kim, Ju *et al.*, 2010) and Alzheimer's (Shih, Chan *et al.*, 2010). Moreover, ANT have beneficial effects on memory and cognition (Shukitt-Hale, Cheng *et al.*, 2009; Spencer, 2010), improving the memory in old rats in Morris water maze test (Andres-Lacueva, Shukitt-Hale *et al.*, 2005), in mice on inhibitory avoidance task (Barros, Amaral *et al.*, 2006) and also ameliorate the cognitive performance of elderly humans (Krikorian, Shidler *et al.*, 2010). Other behavioral findings showed that ANT also have anxiolytic effects in rats and mice in the elevated-plus maze test (Ramirez, Izquierdo *et al.*, 2005; Barros, Amaral *et al.*, 2006).

Acetylcholinesterase (AChE) is an important regulatory enzyme that rapidly hydrolyses the neurotransmitter acetylcholine released by the cholinergic neurons (Paleari, Grozio *et al.*, 2008). Several experimental and clinical studies clearly indicate an undisputed major role of acetylcholine (ACh) in the regulation of cognitive functions (Blokland, 1995). However, the biological role of AChE is not limited to cholinergic transmission. AChE has been implicated in several non-cholinergic actions, including cell proliferation (Appleyard, 1994), neurite outgrowth (Chacon, Reyes *et al.*, 2003) and other responses to various insults as stress and amyloid formation (Grisaru, Sternfeld *et al.*, 1999). 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, Marcos *et al.*, 2009; Mata, Berrocal *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, Sanchez *et al.*, 2010; Panayiotidis, Franco *et al.*, 2010). The former enzyme is responsible for the active transport of Na^+ and K^+, and it is necessary to maintain the ionic gradient across membranes and thus it is essential to regultate neuronal excitability (Kaplan, 2002; Jorgensen, Hakansson *et al.*, 2003; Jimenez, Sanchez *et al.*, 2010). It has been reported that Na^+,K^+-ATPase can play a relevant role in neuronal and synaptic plasticity (Glushchenko e Izvarina, 1997; Scuri, Lombardo *et al.*, 2007) and in the regulation of learning and memory performances (Brunelli, Garcia-Gil *et al.*, 1997; Wyse, Bavaresco *et al.*, 2004). The Ca^{2+}-ATPase is one of the most powerful modulators of intracellular Ca^{2+} levels (Casteels, Wuytack *et al.*, 1991; Raeymaekers e Wuytack, 1991; Huang, Nagaraja *et al.*, 2010). The transients 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 (Missiaen, Callewaert *et al.*, 2000; Missiaen, Robberecht *et al.*, 2000; Ruknudin e Lakatta, 2007; Huang, Nagaraja *et al.*, 2010).

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 Alzheimer's disease (Tota, Awasthi *et al.*, 2010) since it mimics many pathological processes in this disease, as impaired brain glucose and energy and leads to progressive deficits in learning and memory (Lannert e Hoyer, 1998; Awasthi, Tota *et al.*, 2010). Furthermore, it was also observed that icv STZ injection increases AChE activity (Lester-Coll, Rivera *et al.*, 2006; Awasthi, Tota *et al.*, 2010), and decreases the activity and expression of Na^+,K^+-ATPase (Chauhan e Siegel, 1997a; b; Chauhan, Lee *et al.*, 1997). Moreover, it was shown an upregulation of Ca^{2+}-ATPase in the brain of AD patients (Berrocal, Marcos *et al.*, 2009; Mata, Berrocal *et al.*, 2011).

Considering that AD is the most prevalent neurodegenerative disease worldwide in older adults, the search for preventive compounds for this disease is of great social importance. Since ANT have an important function as

antioxidant, neuroprotective and anxiolytic compounds, in this study we investigated whether this natural compound has the ability to prevent memory deficits found in a model of sporadic dementia induced by icv administration of STZ. We also evaluated the levels of nitrite/nitrate and the activities of enzymes important for neurotransmission such as AChE, Na^+,K^+-ATPase and Ca^{2+}-ATPase, which are known to be altered in Alzheimer's disease.

2. Material and Methods

2.1. Chemicals

Acetylthiocholine, Trizma Base, Acetonitrile, Percoll, Coomassie Brilliant Blue G and Streptozotocyn (STZ) were purchased from Sigma Chemical Co (St Luis, MO, USA). Anthocyanins was 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.

2.2 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 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 NIH Guide for Care and Use of Laboratory Animals, and Brazilian Society for Neuroscience and Behavior (SBNeC) recommendations for animal care. This work was approved by the ethical committee of Federal University of Santa Maria (23081.003601/2012-63).

2.3. Administration of drugs to animals

2.3.1. Intracerebroventricular (icv) injection of streptozotocin

Adult male Wistar rats (300–350g) were anaesthetized with thiopental (180mg/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 lateral ventricle (Paxinos e Watson, 1986) were measured accurately as anterio-posterior -0.8mm, lateral 1.5mm and dorso-ventral, -4.0mm 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) was dissolved in citrate buffer (pH 4.4) (Tiwari, Kuhad *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 were given icv injection of same volume of citrate buffer as in STZ treated (Scheme 1).

2.3.2 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 a.m.). The dose of anthocyanin was chosen on the basis of previous studies indicating neuroprotection (Saija, Princi *et al.*, 1990; Manach, Scalbert *et al.*, 2004; Varadinova, Docheva-Drenska *et al.*, 2009; Gutierres, J. M., Carvalho, F. B. *et al.*, 2012). The control groups received only vehicle (2ml/kg gavage of saline, daily per 7 days).

2.4 Behavioral procedure

2.4.1 Inhibitory avoidance task

At the last day of anthocyanin treatment (7^{th} day), the animals were subjected to training in a step-down inhibitory avoidance apparatus as previously described (Rubin, Boemo *et al.*, 2000), after that the animals received icv-STZ (3 mg/kg). Next, 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 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 rat was 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 300s was established.

2.4.2 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, Jessié Martins, Carvalho, Fabiano Barbosa *et al.*, 2012).

2.4.3 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 (Rubin, Albach *et al.*, 2000; Berlese, Sauzem *et al.*, 2005). The animals were placed on the grid and allowed a 3 min habituation period before the start of a series of shocks (1s) 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.

2.4.4 Elevated plus maze task

Anxiolytic-like behavior was evaluated using the task of the elevated plus maze as previously described (Frussa-Filho, Barbosa-Junior *et al.*, 1999; Rubin, Albach *et al.*, 2000). The apparatus consists of a wooden structure raised to 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 an open arm. The animal had 5 minutes to explore the apparatus, and the time spent and the number of entries in open and closed-arms were recorded. The apparatus was thoroughly cleaned with 30% ethanol between each session.

2.5 Brain tissue preparation

After behavioral tests, the animals were anesthetized under 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 10mM, 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, Jessié Martins, Carvalho, Fabiano Barbosa *et al.*, 2012). Protein was determined previously in a strip that varied for each structure: cerebral cortex (0.7 mg/ml) and hippocampus (0.8 mg/ml), as determined by the Coomassie blue method as previously described (Bradford, 1976), using bovine serum albumin as standard solution.

2.6 Isolation of synaptosomes with a discontinuous Percoll gradient

Synaptosomes were isolated essentially as previously described (Nagy e Delgado-Escueta, 1984), with a minor modification (Gutierres, Kaizer *et al.*, 2012b) using a discontinuous Percoll gradient. The cerebral cortex, hippocampus and 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 1,000xg 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 0º-4º throughout the procedure and used to measure Ca^{2+}-ATPase and AChE activities.

2.7. Assay of Lactate Desydrogenase (LDH)

The integrity of the synaptosomes 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, Brasil).

2.8. Determination of AChE activity in brain

The AChE enzymatic assay was determined by a modification of the spectrophotometric method (Rocha, Emanuelli *et al.*, 1993) as previously described (Ellman, Courtney *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 2min 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.

2.9. 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′), 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, Richetti *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.

.

2.10. Na^+,K^+-ATPase activity measurement

Na^+,K^+-ATPase activity was measured as previously described (Wyse, Streck *et al.*, 2000) with minor modifications (Carvalho, Mello *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 37ºC, 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 e 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.

2.11. Ca^{2+}-ATPase activity measurement

Ca^{2+}-ATPase activity was measured as previously described (Rohn, Hinds *et al.*, 1993) with minor modifications (Trevisan, Maldaner *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 37ºC, 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 e 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.

2.12 [H^3] Flunitrazepam specific binding

2.12.1 Membrane preparation

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 1,000 X *g* for 10 min at 4°C. The resulting supernatant was centrifuged at 16,000 X *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.

2.12.2 Radioligand binding assay

[^{3}H] flunitrazepam binding to benzodiazepinic site of $GABA_A$ receptors was determined as previously described (Vogel, Frye *et al.*, 1980), 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 X *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), 0.5 mg of protein membrane and essential oil of lavender or orange (1, 10 and 100 µg/ml). Diazepam (0.1 µM) was used as a positive control. Incubation was started by adding 1 nM [^{3}H] flunitrazepam (85,8Ci/mmol), and run at 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.

2.13. Assay of NOx (NO2 plus NO3) as a marker of NO synthesis

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

2.14. 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).

2.15. 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* following 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.

3. Results

3.1 Behavioral tests

3.1.1 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 (STZ+ANT). Figure 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; $P< 0.01$; Figure 1D]. Statistical analysis of the data obtained during training showed no difference between the different groups (Figure 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*; Figure 1A) or rearing (F (3,42)=1.82, *P>0.05*; Figure 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*]

3.1.2 Effect of STZ and anthocyanin treatment on anxiolytic-like behavior

Figure 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;* Figure 2D] and time in closed arms [F (1,41)= 4.925; *P<0.05;* Figure 2C], revealing that treatment with ANT prevented the anxiolytic like-behavior induced by STZ. However, no significant differences in the number of entries in open arms [F (1,41)= 0.279; *P>0.05;* Figure 2B] and in the number of entries in all arms [F (1,41)= 0.68; *P>0.05;* Figure 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.

3.2 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 binds to the benzodiazepinic site for $GABA_A$ receptor. The results presented in Figure 3 reveal that the ANT (100µM) reduced by 43% the [^{3}H] flunitrazepam binding to benzodiazepinic site of $GABA_A$ receptors [F (2,17)= 47.890; *P<0.0001*] and this result demonstrates that ANT can bind to $GABA_A$ receptor.

3. 3 Activity and expression of acetylcholinesterase

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

Figure 5 shows the effect of ANT and STZ on the AChE expression in 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*].

3.3.2 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. Figure 4 shows the effect of ANT and STZ on the AChE activity in 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 S1 fraction of cerebral cortex [F= (1,28)= 7.973; *P<0.05*] and hippocampus [F (1,28)= 4.995; *P<0.05*] (Figure 4A) induced by icv-STZ.

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 cerebral cortex [F (1,28)= 4.760; *P<0.05*] and hippocampus [F (1,28)= 8. 434; *P<0.01*](Figure 4B) induced by icv-STZ.

3.4 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. Figure 6 shows the effect of ANT and STZ on the activity of Na^+,K^+-ATPase and Ca^{2+}-ATPase in 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 (Figure 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 (Figure 6A).

3.5 NOx levels determination

Anthocyanins are known by their antioxidant properties, so in this set of experiments we investigated if ANT alters nitrite plus nitrate in the brain of rats. Figure 7 shows the effect of ANT and STZ on the NOx levels production in 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 cerebral cortex [F (1,28)= 8.583; *P<0.05*] and hippocampus [F (1,28)= 23.350; *P<0.0001*] induced by icv-STZ.

3.6 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 CTRL group, 102.66±2.13 ANT group, 110.44 ± 1.92 STZ group and 106.77±2.09 STZ+ANT group, respectively, indicating that the dose was sub diabetogenic (Table 1).

4. Discussion

Anthocyanins are flavonoids found in fruits and fruit juices, and have the capacity to improve memory (Harborne e Williams, 2001; Williams e Grayer, 2004; Williams, El Mohsen *et al.*, 2008). Increasing evidences have demonstrated that anthocyanins are able to improve memory of old rats in Morris water maze (Andres-Lacueva, Shukitt-Hale *et al.*, 2005), and of mice in the inhibitory avoidance task (Barros, Amaral *et al.*, 2006) and eldery humans (Krikorian, Shidler *et al.*, 2010). Furthermore, a 2-month dietary supplementation of rats with blueberries prevented deficits in learning performance induced by bilateral hippocampal injections of kainic acid, and the loss of CA1 pyramidal neurons (Duffy, Spangler *et al.*, 2008). In line with this view, studies with the Cyanidin-3-O-glucopyranoside (Cy-3G), an isolated anthocyanin abundant in colorful vegetables and fruits, has been reported to be a neuroprotective phytochemical compound against the Aβ peptide-mediated neurotoxicity in SH-SY5Y cells (Tarozzi, Marchesi *et al.*, 2005; Tarozzi, Morroni *et al.*, 2007; Tarozzi, Merlicco *et al.*, 2008; Tarozzi, Morroni *et al.*, 2010). This compound also reduces cerebral ischemia damage and age-related neuronal deficits in rats (Shin, Park *et al.*, 2006).

Since a large number of evidences suggest the neuroprotective role of anthocyanins, we investigated whether this natural compound is able to prevent the alterations found in a model of sporadic dementia of Alzheimer's type (SDAT) induced by icv-STZ injection. Intracerebroventricular administration of STZ (3mg/kg), a subdiabetogenic dose to rodents has been reported to model SDAT (Lester-Coll, Rivera *et al.*, 2006; Grunblatt, Salkovic-Petrisic *et al.*, 2007), causing a progressive memory impairment and synaptic loss and dysfunction (Lannert e Hoyer, 1998; Pinton, Da Rocha *et al.*, 2010). Our results have shown that icv-STZ impaired the acquisition of memory in rats trained in the inhibitory avoidance task. Interestingly, in this study we found out that a dose of 200 mg/kg of ANT *per se*, during 7 days, did not affect the memory of rats, but it prevented the memory deficits induced by icv-STZ (Figure. 1 D), as assessed by the inhibitory avoidance task. Furthermore, previous studies from our laboratory demonstrated that the dose of 200mg/kg anthocyanins antagonized scopolamine-induced performance deficits in rats in the inhibitory avoidance task (Gutierres, J. M., Carvalho, F. B. *et al.*, 2012) suggesting that anthocyanins

have a close interaction with cholinergic system. The daily intake of anthocyanin chosen for this study (200mg/kg) is in agreement with consumption in residents of the United States population (Manach, Scalbert *et al.*, 2004; Wang e Stoner, 2008). In line with this, previous studies also indicate that a daily intake of 200 mg/kg of anthocyanins has protective effects in rats, mice and humans (Heinonen, 2001; Choi, Ok *et al.*, 2007; Bao, Yao, Tsi *et al.*, 2008; Bao, Yao, Yau *et al.*, 2008) and improve learning and memory in female rats ovarioctomized (Varadinova, Docheva-Drenska *et al.*, 2009).

A major concern in shock motivated learning tests, particularly in those that investigate the effect of drugs given before the acquisition test, is whether pharmacological treatments affect locomotor activities or motivational aspects of learning, such as shock sensitivity. To rule out this possibility, immediately after inhibitory avoidance test, the animals were subjected to an open-field test which is widely used for evaluating motor abnormalities (Belzung e Griebel, 2001). The open field session revealed that the treatment with icv-STZ or ANT did not alter spontaneous locomotor activity as the animals showed a similar number of crossing or rearing responses (Figure 1 A, B). Moreover, we observed that the rats of different groups did not show altered shock sensitivity (Table 2), as verified by their similar flinch, jump and vocalization thresholds. Thus, 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 to learning and memory evaluation, we also measured the anxiolytic-like behavior by the elevated plus maze task, commonly used to study anxiety-related behavior in rodents (Belzung e Griebel, 2001). Our results showed a higher anxiety in icv-STZ group (3 mg/kg) (Figure 2 C; D) which is in accordance with previous observations that mice subjected to icv-STZ, in short time (7days) and long time (21 days) treatments, have a rise increase in anxious behavior (Pinton, Da Rocha *et al.*, 2011). Although we found an anxiolytic effect of treatment with ANT *per se* for the time in closed arm, there was no changed the number of entries and time spent in open arms, which is the parameter most commonly used to represent an anxiolytic effect. In this study, we used a dose of 200 mg/kg based on previous studies showing beneficial effects on learning and memory (Varadinova, Docheva-Drenska *et al.*, 2009; Gutierres, J. M., Carvalho, F. B. *et al.*, 2012). However, we suggest to

investigate anxiolytic effects of anthocyanins per se would be important to select a range of doses. Additionally, the dose chosen in this study (200 mg/kg), was able to prevent the anxiogenic behavior caused by icv-STZ administration (Figure 2 C and D). We believe that the mechanism by which ANT plays anxiolytic effect is related with the affinity of this compound to $GABA_A$ receptor, because the ANT significantly displace the specific binding of [^{3}H] flunitrazepam to benzodiazepinic site of this receptor (Figure 3). This work is the first to describe a specific location where this compound may act to promote an anxiolytic effect, suggesting that anthocyanins may be considered an important pharmacological agent in situations of anxiety.

The pivotal role of cholinergic system in memory is further underlined by use of acetylcholine esterase inhibitors in AD to prevent memory decline. In this study, we found that icv-STZ group showed an increase in AChE activity in supernatant and synaptosomes in relation to all tested groups (Figure 4). This finding is in conformity with the previous studies showing increase in AChE expression (Lester-Coll, Rivera *et al.*, 2006) and activity upon icv-STZ administration (Tota, Kamat *et al.*, 2009; Awasthi, Tota *et al.*, 2010; Tota, Awasthi *et al.*, 2010). In addition, icv-STZ also caused cholinergic deficiency supported by reduced cholineacetyltransferase (ChAT) activity in hippocampus of rats (Blokland e Jolles, 1993; Sharma e Gupta, 2001b; a; Sonkusare, Srinivasan *et al.*, 2005). It is possible that the increased AChE activity is due to a reduction in energy metabolism and oxidative stress leading to cognitive dysfunction by inhibiting the synthesis of adenosine triphosphate (ATP) and acetyl-CoA (Blokland e Jolles, 1993). Moreover, the impairment in insulin signalling and increased oxidative stress induced by icv-STZ administration was associated with the upregulation of AChE in the brain of rats (De La Monte, Tong *et al.*, 2006; Lester-Coll, Rivera *et al.*, 2006). Our research group has demonstrated that intraperitoneal STZ administration, a model of diabetes type 2; causes a significant increase in AChE activity in synaptosomes of cerebral cortex of rats (Schmatz, Mazzanti *et al.*, 2009). In the present study, we observed that ANT was able to prevent the AChE upregulation in hippocampus and cerebral cortex of icv-STZ animals, although this compound *per se* had no effect on AChE activity. This effect of ANT can be attributed to its potent antioxidant effect.

Agonists of cholinergic receptors and inhibitors of AChE have been extensively used in order to increase endogenous acetylcholine (ACh) levels and thus overcome cognitive deficits (Lane, Potkin *et al.*, 2006). AChE metabolizes ACh to choline and acetyl-CoA. AChE exists into different molecular forms, which can be distinguished on the basis of their shapes, e.g., collagen-tailed asymmetric forms and globular (G) forms (Lane, Potkin *et al.*, 2006). Although few studies have shown that the isoforms of AChE may be differentially expressed in different brain regions (Malatova, Nicak *et al.*, 1980; Zakut, Matzkel *et al.*, 1985; Lane, Potkin *et al.*, 2006), and that these isoforms can be considered important markers of AD (Kasa, Rakonczay *et al.*, 1997; Shen, 2004; Lane, Potkin *et al.*, 2006), it is known that AChE activity in homogenate (S1) shows the total AChE activity (different isoforms associated), while the synaptosomal fractions are re-sealed nerve terminal with a greater amount of membrane-bound isoforms G4 (Mazzanti, Spanevello *et al.*, 2006). Our work showed that treatment with STZ or ANT did not affect a specific AChE isoform, because the AChE activities measured in S1 and synaptosomes fractions were similar.

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 (Gether, Andersen *et al.*, 2006; Gouaux, 2009; Benarroch, 2011). 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 behaviours of rats (Dos Reis, De Oliveira *et al.*, 2002; Lingrel, Williams *et al.*, 2007; Moseley, Williams *et al.*, 2007). *In vitro* studies showed that the inhibitor of Na^+,K^+-ATPase, *ouabain*, increases the Ca^{2+} influx into slices of rats brain (Fujisawa, Kajikawa *et al.*, 1965), induces the release of glutamate by reverse transport of Na^+ (Li e Stys, 2001) and cause excitotoxicity in hippocampal neurons (Lees, Lehmann *et al.*, 1990). Corroborating these findings, our study showed that icv-STZ administration decreased Na^+, K^+-ATPase activity and increased Ca^{2+}-ATPase activity (Figure 5), suggesting that a disturbance in the eletrolitic concentrations of Na^+ and Ca^{2+} could lead to

excitotoxicity and neuronal death in icv-STZ induced experimental model of Alzheimer's type.

Furthermore, it was also found that the inhibition of Na^+,K^+-ATPase increase NMDA-mediated currents in the hippocampus (Zhang, Guo *et al.*, 2011). It is known that NMDA receptor activation increases the nitric oxide (NO) synthesis by increasing nitric oxide synthase activity (NOS) (Sattler, Xiong *et al.*, 1999; Prast e Philippu, 2001). NO is a retrograde messenger which diffuses through the cellular membranes and activation of guanylate cyclase and PKG (East e 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, Bulygina *et al.*, 2003; Boldyrev, Bulygina *et al.*, 2004; Carvalho, Mello *et al.*, 2012). Our results, show that icv-STZ administration increases the nitrate/nitrite levels (Figure 6), markers of NO synthesis, 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 (Takeguchi, Honegger *et al.*, 1976; Lipton, Choi *et al.*, 1993; Lipton, Singel *et al.*, 1994; Boldyrev e Bulygina, 1997; Boldyrev, Bulygina *et al.*, 1997); 2- activation of signaling pathway related with NOS/cGMP/PKG (Carvalho, Mello *et al.*, 2012).

Increasing evidences have demonstrated a correlation between the consumption of fresh fruits and vegetables with the prevention, delay or onset of chronic degenerative diseases including cancer (Juranic e Zizak, 2005), diabetes mellitus (Matsui, Ogunwande *et al.*, 2006) and AD (Bishayee, Haznagy-Radnai *et al.*, 2010; Darvesh, Carroll *et al.*, 2010). These studies have stated that in addition to ANT antioxidant effects, these compounds decrease the levels of NO (Blokland e Jolles, 1993; Juranic e Zizak, 2005). The data presented in this paper demonstrates that ANT (200 mg/kg) prevented the augment 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, Rossi *et al.*, 2006; Wang, Xia *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, Pancewicz *et al.*, 2007; Valko, Leibfritz *et al.*, 2007). The Na^+, K^+-ATPase is a transmembrane enzyme sensitive to ROS and RNS (Wyse, Bavaresco *et al.*, 2001; Dos Reis, De Oliveira *et al.*, 2002; Franzon, Lamers *et al.*, 2003), while the Ca^{2+}-ATPase is an enzyme sensitive to intracellular Ca^{2+} levels (Casteels, Wuytack *et al.*, 1991; Raeymaekers e Wuytack, 1991; Huang, Nagaraja *et al.*, 2010). The latter enzyme is the major modulator of the intracellular concentrations of this second messenger, and increases in the activity of Ca^{2+}-ATPase could be a compensatory mechanism to remove an excess of Ca^{2+} ions, and this can be the result of oxidative damage in the Na^+, K^+-ATPase enzyme induced by icv-STZ injection. Thus, the antioxidant activity of the ANT may be related with a protective effect on the dysfunction of these enzymes.

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, Ansaldo *et al.*, 2000). Furthermore, the activation of $GABA_A$ receptors protects neurons against Aβ toxicity in AD-affected regions in mammalian brain (Paula-Lima, De Felice *et al.*, 2005).

Thus, since up-regulation of cholinergic system, memory impairment associated with anxiogenic behavior has been related with pathophysiology of AD, our results suggest that the ANT could exert beneficial actions, preventing the increase in AChE activity and memory loss induced by icv-STZ, a model of SDAT. 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.

Acknowledgments

This study was supported by the Christian Hansen LTDA, Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Rio Grande do Sul (FAPERGS).

Legends

Table 1. Structural identification of anthocyanins.

Table 2. The primers used for the gene amplification

Table 3. Effects of anthocyanin (200mg/kg) treatment and icv-STZ (3mg/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.

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.

Scheme 1. Exposure design

Figure 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 was 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 group. *Denotes *P<0.05* as compared to the others 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*.

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

Figure 3. Anthocyanin (100µM) reduced the specific [3H] flunitrazepam binding to 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.

Figure 4. AChE activity (A) in supernatant fraction and (B) synaptosomes fraction of 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 group. **P<0.05* compared with the others groups; ANOVA (*two-way*) followed by Tukey test.

Figure 5. Relative gene expression pattern of AChE (A) in 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 group. *P<0.05 compared with the others groups; ANOVA (two-way) followed by Tukey test. No significative changes were observed between groups.

Figure 6. Na^+,K^+-ATPase (A) and Ca^+-ATPase (B) activity in 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 group. **P<0.05* compared with the others groups; ANOVA (*two-way*) followed by Tukey test.

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

References

[1] Williams, C. A., Grayer, R. J. Anthocyanins and other flavonoids. Natural product reports. 2004,21:539-73.
[2] Veitch, N. C., Grayer, R. J. Flavonoids and their glycosides, including anthocyanins. Natural product reports. 2011,28:1626-95.
[3] Yoshida, K., Mori, M., Kondo, T. Blue flower color development by anthocyanins: from chemical structure to cell physiology. Natural product reports. 2009,26:884-915.
[4] Kahkonen, M. P., Hopia, A. I., Heinonen, M. Berry phenolics and their antioxidant activity. Journal of agricultural and food chemistry. 2001,49:4076-82.
[5] Kahkonen, M. P., Heinonen, M. Antioxidant activity of anthocyanins and their aglycons. Journal of agricultural and food chemistry. 2003,51:628-33.
[6] Del Rio, D., Borges, G., Crozier, A. Berry flavonoids and phenolics: bioavailability and evidence of protective effects. The British journal of nutrition. 2010,104 Suppl 3:S67-90.
[7] Ke, Z., Liu, Y., Wang, X., Fan, Z., Chen, G., Xu, M., et al. Cyanidin-3-glucoside ameliorates ethanol neurotoxicity in the developing brain. Journal of neuroscience research. 2011,89:1676-84.
[8] Shin, W. H., Park, S. J., Kim, E. J. Protective effect of anthocyanins in middle cerebral artery occlusion and reperfusion model of cerebral ischemia in rats. Life sciences. 2006,79:130-7.
[9] Min, J., Yu, S. W., Baek, S. H., Nair, K. M., Bae, O. N., Bhatt, A., et al. Neuroprotective effect of cyanidin-3-O-glucoside anthocyanin in mice with focal cerebral ischemia. Neurosci Lett. 2011,500:157-61.
[10] Kim, H. G., Ju, M. S., Shim, J. S., Kim, M. C., Lee, S. H., Huh, Y., et al. Mulberry fruit protects dopaminergic neurons in toxin-induced Parkinson's disease models. The British journal of nutrition. 2010,104:8-16.
[11] Shih, P. H., Chan, Y. C., Liao, J. W., Wang, M. F., Yen, G. C. Antioxidant and cognitive promotion effects of anthocyanin-rich mulberry (Morus atropurpurea L.) on senescence-accelerated mice and prevention of Alzheimer's disease. The Journal of nutritional biochemistry. 2010,21:598-605.
[12] Spencer, J. P. The impact of fruit flavonoids on memory and cognition. The British journal of nutrition. 2010,104 Suppl 3:S40-7.
[13] Shukitt-Hale, B., Cheng, V., Joseph, J. A. Effects of blackberries on motor and cognitive function in aged rats. Nutritional neuroscience. 2009,12:135-40.
[14] Andres-Lacueva, C., Shukitt-Hale, B., Galli, R. L., Jauregui, O., Lamuela-Raventos, R. M., Joseph, J. A. Anthocyanins in aged blueberry-fed rats are found centrally and may enhance memory. Nutritional neuroscience. 2005,8:111-20.
[15] Barros, D., Amaral, O. B., Izquierdo, I., Geracitano, L., do Carmo Bassols Raseira, M., Henriques, A. T., et al. Behavioral and genoprotective effects of Vaccinium berries intake in mice. Pharmacology, biochemistry, and behavior. 2006,84:229-34.
[16] Krikorian, R., Shidler, M. D., Nash, T. A., Kalt, W., Vinqvist-Tymchuk, M. R., Shukitt-Hale, B., et al. Blueberry supplementation improves memory in older adults. Journal of agricultural and food chemistry. 2010,58:3996-4000.
[17] Ramirez, M. R., Izquierdo, I., do Carmo Bassols Raseira, M., Zuanazzi, J. A., Barros, D., Henriques, A. T. Effect of lyophilised Vaccinium berries on memory, anxiety and locomotion in adult rats. Pharmacological research : the official journal of the Italian Pharmacological Society. 2005,52:457-62.
[18] Paleari, L., Grozio, A., Cesario, A., Russo, P. The cholinergic system and cancer. Seminars in cancer biology. 2008,18:211-7.

[19] Blokland, A. Acetylcholine: a neurotransmitter for learning and memory? Brain Res Brain Res Rev. 1995,21:285-300.
[20] Appleyard, M. E. Non-cholinergic functions of acetylcholinesterase. Biochem Soc Trans. 1994,22:749-55.
[21] Chacon, M. A., Reyes, A. E., Inestrosa, N. C. Acetylcholinesterase induces neuronal cell loss, astrocyte hypertrophy and behavioral deficits in mammalian hippocampus. J Neurochem. 2003,87:195-204.
[22] Grisaru, D., Sternfeld, M., Eldor, A., Glick, D., Soreq, H. Structural roles of acetylcholinesterase variants in biology and pathology. Eur J Biochem. 1999,264:672-86.
[23] Berrocal, M., Marcos, D., Sepulveda, M. R., Perez, M., Avila, J., Mata, A. M. Altered Ca2+ dependence of synaptosomal plasma membrane Ca2+-ATPase in human brain affected by Alzheimer's disease. The FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2009,23:1826-34.
[24] Mata, A. M., Berrocal, M., Sepulveda, M. R. Impairment of the activity of the plasma membrane Ca(2)(+)-ATPase in Alzheimer's disease. Biochem Soc Trans. 2011,39:819-22.
[25] Jimenez, T., Sanchez, G., Wertheimer, E., Blanco, G. Activity of the Na,K-ATPase alpha4 isoform is important for membrane potential, intracellular Ca2+, and pH to maintain motility in rat spermatozoa. Reproduction. 2010,139:835-45.
[26] Panayiotidis, M. I., Franco, R., Bortner, C. D., Cidlowski, J. A. Ouabain-induced perturbations in intracellular ionic homeostasis regulate death receptor-mediated apoptosis. Apoptosis : an international journal on programmed cell death. 2010,15:834-49.
[27] Kaplan, J. H. Biochemistry of Na,K-ATPase. Annu Rev Biochem. 2002,71:511-35.
[28] Jorgensen, P. L., Hakansson, K. O., Karlish, S. J. Structure and mechanism of Na,K-ATPase: functional sites and their interactions. Annu Rev Physiol. 2003,65:817-49.
[29] Glushchenko, T. S., Izvarina, N. L. Na+,K(+)-ATPase activity in neurons and glial cells of the olfactory cortex of the rat brain during the development of long-term potentiation. Neurosci Behav Physiol. 1997,27:49-52.
[30] Scuri, R., Lombardo, P., Cataldo, E., Ristori, C., Brunelli, M. Inhibition of Na+/K+ ATPase potentiates synaptic transmission in tactile sensory neurons of the leech. Eur J Neurosci. 2007,25:159-67.
[31] Brunelli, M., Garcia-Gil, M., Mozzachiodi, R., Scuri, R., Zaccardi, M. L. Neurobiological principles of learning and memory. Arch Ital Biol. 1997,135:15-36.
[32] Wyse, A. T., Bavaresco, C. S., Reis, E. A., Zugno, A. I., Tagliari, B., Calcagnotto, T., et al. Training in inhibitory avoidance causes a reduction of Na+,K+-ATPase activity in rat hippocampus. Physiol Behav. 2004,80:475-9.
[33] Raeymaekers, L., Wuytack, F. [The Ca(2+)-transport ATPases of smooth muscle]. Verhandelingen - Koninklijke Academie voor Geneeskunde van Belgie. 1991,53:605-28.
[34] Casteels, R., Wuytack, F., Raeymaekers, L., Himpens, B. Ca(2+)-transport ATPases and Ca(2+)-compartments in smooth muscle cells. Zeitschrift fur Kardiologie. 1991,80 Suppl 7:65-8.
[35] Huang, H., Nagaraja, R. Y., Garside, M. L., Akemann, W., Knopfel, T., Empson, R. M. Contribution of plasma membrane Ca ATPase to cerebellar synapse function. World journal of biological chemistry. 2010,1:95-102.

[36] Missiaen, L., Callewaert, G., Parys, J. B., Wuytack, F., Raeymaekers, L., Droogmans, G., et al. [Intracellular calcium: physiology and physiopathology]. Verhandelingen - Koninklijke Academie voor Geneeskunde van Belgie. 2000,62:471-99.
[37] Missiaen, L., Robberecht, W., van den Bosch, L., Callewaert, G., Parys, J. B., Wuytack, F., et al. Abnormal intracellular ca(2+)homeostasis and disease. Cell calcium. 2000,28:1-21.
[38] Ruknudin, A. M., Lakatta, E. G. The regulation of the Na/Ca exchanger and plasmalemmal Ca2+ ATPase by other proteins. Ann N Y Acad Sci. 2007,1099:86-102.
[39] Hoyer, S. Causes and consequences of disturbances of cerebral glucose metabolism in sporadic Alzheimer disease: therapeutic implications. Adv Exp Med Biol. 2004,541:135-52.
[40] Hoyer, S. Glucose metabolism and insulin receptor signal transduction in Alzheimer disease. Eur J Pharmacol. 2004,490:115-25.
[41] Tota, S., Awasthi, H., Kamat, P. K., Nath, C., Hanif, K. Protective effect of quercetin against intracerebral streptozotocin induced reduction in cerebral blood flow and impairment of memory in mice. Behav Brain Res. 2010,209:73-9.
[42] Lannert, H., Hoyer, S. Intracerebroventricular administration of streptozotocin causes long-term diminutions in learning and memory abilities and in cerebral energy metabolism in adult rats. Behav Neurosci. 1998,112:1199-208.
[43] Awasthi, H., Tota, S., Hanif, K., Nath, C., Shukla, R. Protective effect of curcumin against intracerebral streptozotocin induced impairment in memory and cerebral blood flow. Life Sci. 2010,86:87-94.
[44] Lester-Coll, N., Rivera, E. J., Soscia, S. J., Doiron, K., Wands, J. R., de la Monte, S. M. Intracerebral streptozotocin model of type 3 diabetes: relevance to sporadic Alzheimer's disease. Journal of Alzheimer's disease : JAD. 2006,9:13-33.
[45] Chauhan, N., Siegel, G. Differential expression of Na,K-ATPase alpha-isoform mRNAs in aging rat cerebellum. Journal of neuroscience research. 1997,47:287-99.
[46] Chauhan, N., Siegel, G. Na,K-ATPase: increases in alpha1-messenger RNA and decreases in alpha3-messenger RNA levels in aging rat cerebral cortex. Neuroscience. 1997,78:7-11.
[47] Chauhan, N. B., Lee, J. M., Siegel, G. J. Na,K-ATPase mRNA levels and plaque load in Alzheimer's disease. J Mol Neurosci. 1997,9:151-66.
[48] Paxinos, G., Watson, C. The Rat Brain in Stereotaxic Coordinates. Academic Press, San Diego. 1986.
[49] Tiwari, V., Kuhad, A., Bishnoi, M., Chopra, K. Chronic treatment with tocotrienol, an isoform of vitamin E, prevents intracerebroventricular streptozotocin-induced cognitive impairment and oxidative-nitrosative stress in rats. Pharmacology, biochemistry, and behavior. 2009,93:183-9.
[50] Saija, A., Princi, P., D'Amico, N., De Pasquale, R., Costa, G. Effect of Vaccinium myrtillus anthocyanins on triiodothyronine transport into brain in the rat. Pharmacol Res. 1990,22 Suppl 3:59-60.
[51] Manach, C., Scalbert, A., Morand, C., Remesy, C., Jimenez, L. Polyphenols: food sources and bioavailability. The American journal of clinical nutrition. 2004,79:727-47.

[52] Varadinova, M. G., Docheva-Drenska, D. I., Boyadjieva, N. I. Effects of anthocyanins on learning and memory of ovariectomized rats. Menopause. 2009,16:345-9.
[53] Gutierres, J. M., Carvalho, F. B., Schetinger, M. R., Rodrigues, M. V., Schmatz, R., Pimentel, V. C., et al. Protective effects of anthocyanins on the ectonucleotidase activity in the impairment of memory induced by scopolamine in adult rats. Life Sci. 2012,91:1221-8.
[54] Rubin, M. A., Boemo, R. L., Jurach, A., Rojas, D. B., Zanolla, G. R., Obregon, A. D., et al. Intrahippocampal spermidine administration improves inhibitory avoidance performance in rats. Behav Pharmacol. 2000,11:57-61.
[55] Gutierres, J. M., Carvalho, F. B., Rosa, M. M., Schmatz, R., Rodrigues, M., Vieira, J. M., et al. Protective effect of α-Tocopherol on memory deficits and Na+,K+-ATPase and acetylcholinesterase activities in rats with diet-induced hypercholesterolemia. Biomedicine & Aging Pathology. 2012,2:73-80.
[56] Rubin, M. A., Albach, C. A., Berlese, D. B., Bonacorso, H. G., Bittencourt, S. R., Queiroz, C. M., et al. Anxiolytic-like effects of 4-phenyl-2-trichloromethyl-3H-1, 5-benzodiazepine hydrogen sulfate in mice. Braz J Med Biol Res. 2000,33:1069-73.
[57] Berlese, D. B., Sauzem, P. D., Carati, M. C., Guerra, G. P., Stiegemeier, J. A., Mello, C. F., et al. Time-dependent modulation of inhibitory avoidance memory by spermidine in rats. Neurobiol Learn Mem. 2005,83:48-53.
[58] Frussa-Filho, R., Barbosa-Junior, H., Silva, R. H., Da Cunha, C., Mello, C. F. Naltrexone potentiates the anxiolytic effects of chlordiazepoxide in rats exposed to novel environments. Psychopharmacology (Berl). 1999,147:168-73.
[59] Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976,72:248-54.
[60] Nagy, A., Delgado-Escueta, A. V. Rapid preparation of synaptosomes from mammalian brain using nontoxic isoosmotic gradient material (Percoll). J Neurochem. 1984,43:1114-23.
[61] Gutierres, J. M., Kaizer, R. R., Schmatz, R., Mazzanti, C. M., Vieira, J. M., Rodrigues, M. V., et al. alpha-Tocopherol regulates ectonucleotidase activities in synaptosomes from rats fed a high-fat diet. Cell Biochem Funct. 2012.
[62] Rocha, J. B., Emanuelli, T., Pereira, M. E. Effects of early undernutrition on kinetic parameters of brain acetylcholinesterase from adult rats. Acta neurobiologiae experimentalis. 1993,53:431-7.
[63] Ellman, G. L., Courtney, K. D., Andres, V., Jr., Feather-Stone, R. M. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol. 1961,7:88-95.
[64] da Silva, R. S., Richetti, S. K., da Silveira, V. G., Battastini, A. M., Bogo, M. R., Lara, D. R., et al. Maternal caffeine intake affects acetylcholinesterase in hippocampus of neonate rats. International journal of developmental neuroscience : the official journal of the International Society for Developmental Neuroscience. 2008,26:339-43.
[65] Wyse, A. T., Streck, E. L., Barros, S. V., Brusque, A. M., Zugno, A. I., Wajner, M. Methylmalonate administration decreases Na+,K+-ATPase activity in cerebral cortex of rats. Neuroreport. 2000,11:2331-4.
[66] Carvalho, F. B., Mello, C. F., Marisco, P. C., Tonello, R., Girardi, B. A., Ferreira, J., et al. Spermidine decreases Na(+),K(+)-ATPase activity through NMDA receptor and protein kinase G activation in the hippocampus of rats. Eur J Pharmacol. 2012,684:79-86.

[67] Fiske, C. H., Subbarow, Y. The Nature of the "Inorganic Phosphate" in Voluntary Muscle. Science. 1927,65:401-3.
[68] Rohn, T. T., Hinds, T. R., Vincenzi, F. F. Ion transport ATPases as targets for free radical damage. Protection by an aminosteroid of the Ca2+ pump ATPase and Na+/K+ pump ATPase of human red blood cell membranes. Biochem Pharmacol. 1993,46:525-34.
[69] Trevisan, G., Maldaner, G., Velloso, N. A., Sant'Anna Gda, S., Ilha, V., Velho Gewehr Cde, C., et al. Antinociceptive effects of 14-membered cyclopeptide alkaloids. Journal of natural products. 2009,72:608-12.
[70] Vogel, R. A., Frye, G. D., Wilson, J. H., Kuhn, C. M., Koepke, K. M., Mailman, R. B., et al. Attenuation of the effects of punishment by ethanol: comparisons with chlordiazepoxide. Psychopharmacology (Berl). 1980,71:123-9.
[71] Miranda, K. M., Espey, M. G., Wink, D. A. A rapid, simple spectrophotometric method for simultaneous detection of nitrate and nitrite. Nitric Oxide. 2001,5:62-71.
[72] Harborne, J. B., Williams, C. A. Anthocyanins and other flavonoids. Natural product reports. 2001,18:310-33.
[73] Williams, C. M., El Mohsen, M. A., Vauzour, D., Rendeiro, C., Butler, L. T., Ellis, J. A., et al. Blueberry-induced changes in spatial working memory correlate with changes in hippocampal CREB phosphorylation and brain-derived neurotrophic factor (BDNF) levels. Free radical biology & medicine. 2008,45:295-305.
[74] Duffy, K. B., Spangler, E. L., Devan, B. D., Guo, Z., Bowker, J. L., Janas, A. M., et al. A blueberry-enriched diet provides cellular protection against oxidative stress and reduces a kainate-induced learning impairment in rats. Neurobiol Aging. 2008,29:1680-9.
[75] Tarozzi, A., Merlicco, A., Morroni, F., Franco, F., Cantelli-Forti, G., Teti, G., et al. Cyanidin 3-O-glucopyranoside protects and rescues SH-SY5Y cells against amyloid-beta peptide-induced toxicity. Neuroreport. 2008,19:1483-6.
[76] Tarozzi, A., Morroni, F., Hrelia, S., Angeloni, C., Marchesi, A., Cantelli-Forti, G., et al. Neuroprotective effects of anthocyanins and their in vivo metabolites in SH-SY5Y cells. Neuroscience letters. 2007,424:36-40.
[77] Tarozzi, A., Marchesi, A., Hrelia, S., Angeloni, C., Andrisano, V., Fiori, J., et al. Protective effects of cyanidin-3-O-beta-glucopyranoside against UVA-induced oxidative stress in human keratinocytes. Photochemistry and photobiology. 2005,81:623-9.
[78] Tarozzi, A., Morroni, F., Merlicco, A., Bolondi, C., Teti, G., Falconi, M., et al. Neuroprotective effects of cyanidin 3-O-glucopyranoside on amyloid beta (25-35) oligomer-induced toxicity. Neurosci Lett. 2010,473:72-6.
[79] Grunblatt, E., Salkovic-Petrisic, M., Osmanovic, J., Riederer, P., Hoyer, S. Brain insulin system dysfunction in streptozotocin intracerebroventricularly treated rats generates hyperphosphorylated tau protein. J Neurochem. 2007,101:757-70.
[80] Pinton, S., da Rocha, J. T., Zeni, G., Nogueira, C. W. Organoselenium improves memory decline in mice: involvement of acetylcholinesterase activity. Neurosci Lett. 2010,472:56-60.
[81] Wang, L. S., Stoner, G. D. Anthocyanins and their role in cancer prevention. Cancer letters. 2008,269:281-90.
[82] Bao, L., Yao, X. S., Yau, C. C., Tsi, D., Chia, C. S., Nagai, H., et al. Protective effects of bilberry (Vaccinium myrtillus L.) extract on restraint stress-

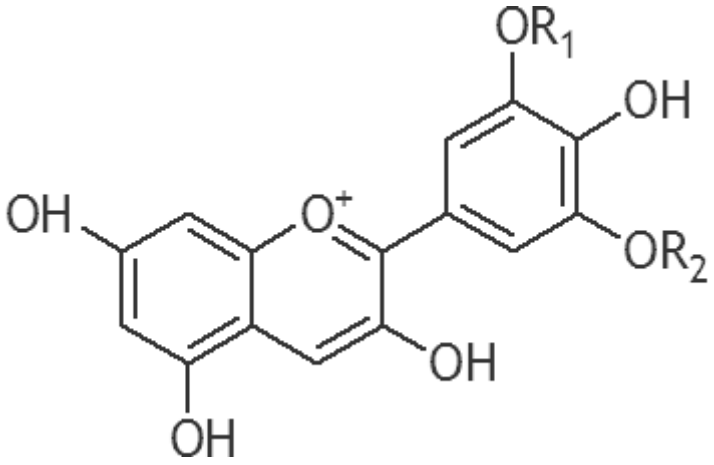
induced liver damage in mice. Journal of agricultural and food chemistry. 2008,56:7803-7.
[83] Bao, L., Yao, X. S., Tsi, D., Yau, C. C., Chia, C. S., Nagai, H., et al. Protective effects of bilberry (Vaccinium myrtillus L.) extract on KBrO3-induced kidney damage in mice. Journal of agricultural and food chemistry. 2008,56:420-5.
[84] Choi, E. H., Ok, H. E., Yoon, Y., Magnuson, B. A., Kim, M. K., Chun, H. S. Protective effect of anthocyanin-rich extract from bilberry (Vaccinium myrtillus L.) against myelotoxicity induced by 5-fluorouracil. Biofactors. 2007,29:55-65.
[85] Heinonen, M. Anthocyanins as dietary antioxidants. Third international conference on natural antioxidants and anticarcinogens in food, health and disease (NADH). 2001:6-9.
[86] Belzung, C., Griebel, G. Measuring normal and pathological anxiety-like behaviour in mice: a review. Behav Brain Res. 2001,125:141-9.
[87] Pinton, S., da Rocha, J. T., Gai, B. M., Nogueira, C. W. Sporadic dementia of Alzheimer's type induced by streptozotocin promotes anxiogenic behavior in mice. Behav Brain Res. 2011,223:1-6.
[88] Tota, S., Kamat, P. K., Awasthi, H., Singh, N., Raghubir, R., Nath, C., et al. Candesartan improves memory decline in mice: involvement of AT1 receptors in memory deficit induced by intracerebral streptozotocin. Behav Brain Res. 2009,199:235-40.
[89] Blokland, A., Jolles, J. Spatial learning deficit and reduced hippocampal ChAT activity in rats after an ICV injection of streptozotocin. Pharmacology, biochemistry, and behavior. 1993,44:491-4.
[90] Sharma, M., Gupta, Y. K. Intracerebroventricular injection of streptozotocin in rats produces both oxidative stress in the brain and cognitive impairment. Life Sci. 2001,68:1021-9.
[91] Sharma, M., Gupta, Y. K. Effect of chronic treatment of melatonin on learning, memory and oxidative deficiencies induced by intracerebroventricular streptozotocin in rats. Pharmacology, biochemistry, and behavior. 2001,70:325-31.
[92] Sonkusare, S., Srinivasan, K., Kaul, C., Ramarao, P. Effect of donepezil and lercanidipine on memory impairment induced by intracerebroventricular streptozotocin in rats. Life Sci. 2005,77:1-14.
[93] de la Monte, S. M., Tong, M., Lester-Coll, N., Plater, M., Jr., Wands, J. R. Therapeutic rescue of neurodegeneration in experimental type 3 diabetes: relevance to Alzheimer's disease. Journal of Alzheimer's disease : JAD. 2006,10:89-109.
[94] Schmatz, R., Mazzanti, C. M., Spanevello, R., Stefanello, N., Gutierres, J., Maldonado, P. A., et al. Ectonucleotidase and acetylcholinesterase activities in synaptosomes from the cerebral cortex of streptozotocin-induced diabetic rats and treated with resveratrol. Brain research bulletin. 2009,80:371-6.
[95] Lane, R. M., Potkin, S. G., Enz, A. Targeting acetylcholinesterase and butyrylcholinesterase in dementia. Int J Neuropsychopharmacol. 2006,9:101-24.
[96] Zakut, H., Matzkel, A., Schejter, E., Avni, A., Soreq, H. Polymorphism of acetylcholinesterase in discrete regions of the developing human fetal brain. J Neurochem. 1985,45:382-9.
[97] Malatova, Z., Nicak, A., Marsala, J. Acetylcholinesterase activity and its molecular forms in selected regions of the dog's nervous system. Physiologia Bohemoslovaca. 1980,29:415-22.
[98] Kasa, P., Rakonczay, Z., Gulya, K. The cholinergic system in Alzheimer's disease. Prog Neurobiol. 1997,52:511-35.

[99] Shen, Z. X. Brain cholinesterases: I. The clinico-histopathological and biochemical basis of Alzheimer's disease. Med Hypotheses. 2004,63:285-97.
[100] Mazzanti, C. M., Spanevello, R. M., Pereira, L. B., Goncalves, J. F., Kaizer, R., Correa, M., et al. Acetylcholinesterase activity in rats experimentally demyelinated with ethidium bromide and treated with interferon beta. Neurochem Res. 2006,31:1027-34.
[101] Benarroch, E. E. Na+, K+-ATPase: functions in the nervous system and involvement in neurologic disease. Neurology. 2011,76:287-93.
[102] Gether, U., Andersen, P. H., Larsson, O. M., Schousboe, A. Neurotransmitter transporters: molecular function of important drug targets. Trends Pharmacol Sci. 2006,27:375-83.
[103] Gouaux, E. Review. The molecular logic of sodium-coupled neurotransmitter transporters. Philosophical transactions of the Royal Society of London. Series B, Biological sciences. 2009,364:149-54.
[104] dos Reis, E. A., de Oliveira, L. S., Lamers, M. L., Netto, C. A., Wyse, A. T. Arginine administration inhibits hippocampal Na(+),K(+)-ATPase activity and impairs retention of an inhibitory avoidance task in rats. Brain Res. 2002,951:151-7.
[105] Lingrel, J. B., Williams, M. T., Vorhees, C. V., Moseley, A. E. Na,K-ATPase and the role of alpha isoforms in behavior. J Bioenerg Biomembr. 2007,39:385-9.
[106] Moseley, A. E., Williams, M. T., Schaefer, T. L., Bohanan, C. S., Neumann, J. C., Behbehani, M. M., et al. Deficiency in Na,K-ATPase alpha isoform genes alters spatial learning, motor activity, and anxiety in mice. J Neurosci. 2007,27:616-26.
[107] Fujisawa, H., Kajikawa, K., Ohi, Y., Hashimoto, Y., Yoshida, H. Movement of radioactive calcium in brain slices and influences on it of protoveratrine, ouabain, potassium chloride and cocaine. Jpn J Pharmacol. 1965,15:327-34.
[108] Li, S., Stys, P. K. Na(+)-K(+)-ATPase inhibition and depolarization induce glutamate release via reverse Na(+)-dependent transport in spinal cord white matter. Neuroscience. 2001,107:675-83.
[109] Lees, G. J., Lehmann, A., Sandberg, M., Hamberger, A. The neurotoxicity of ouabain, a sodium-potassium ATPase inhibitor, in the rat hippocampus. Neurosci Lett. 1990,120:159-62.
[110] Zhang, L., Guo, F., Su, S., Guo, H., Xiong, C., Yin, J., et al. Na(+) /K(+) -ATPase inhibition upregulates NMDA-evoked currents in rat hippocampal CA1 pyramidal neurons. Fundam Clin Pharmacol. 2011.
[111] Prast, H., Philippu, A. Nitric oxide as modulator of neuronal function. Prog Neurobiol. 2001,64:51-68.
[112] Sattler, R., Xiong, Z., Lu, W. Y., Hafner, M., MacDonald, J. F., Tymianski, M. Specific coupling of NMDA receptor activation to nitric oxide neurotoxicity by PSD-95 protein. Science. 1999,284:1845-8.
[113] East, S. J., Garthwaite, J. NMDA receptor activation in rat hippocampus induces cyclic GMP formation through the L-arginine-nitric oxide pathway. Neurosci Lett. 1991,123:17-9.
[114] Boldyrev, A., Bulygina, E., Gerassimova, O., Lyapina, L., Schoner, W. Functional relationship between Na/K-ATPase and NMDA-receptors in rat cerebellum granule cells. Biochemistry (Mosc). 2004,69:429-34.
[115] Boldyrev, A., Bulygina, E., Carpenter, D., Schoner, W. Glutamate receptors communicate with Na+/K+-ATPase in rat cerebellum granule cells: demonstration of differences in the action of several metabotropic and

ionotropic glutamate agonists on intracellular reactive oxygen species and the sodium pump. J Mol Neurosci. 2003,21:213-22.
[116] Boldyrev, A. A., Bulygina, E. R., Kramarenko, G. G., Vanin, A. F. Effect of nitroso compounds on Na/K-ATPase. Biochim Biophys Acta. 1997,1321:243-51.
[117] Boldyrev, A. A., Bulygina, E. R. Na/K-ATPase and oxidative stress. Ann N Y Acad Sci. 1997,834:666-8.
[118] Lipton, S. A., Choi, Y. B., Pan, Z. H., Lei, S. Z., Chen, H. S., Sucher, N. J., et al. A redox-based mechanism for the neuroprotective and neurodestructive effects of nitric oxide and related nitroso-compounds. Nature. 1993,364:626-32.
[119] Lipton, S. A., Singel, D. J., Stamler, J. S. Nitric oxide in the central nervous system. Progress in brain research. 1994,103:359-64.
[120] Takeguchi, C. A., Honegger, U. E., Holland, W. W., Titus, E. O. Evidence for subclasses of SH groups in (Na++K+)-ATPase. Life Sci. 1976,19:797-805.
[121] Juranic, Z., Zizak, Z. Biological activities of berries: from antioxidant capacity to anti-cancer effects. Biofactors. 2005,23:207-11.
[122] Matsui, T., Ogunwande, I. A., Abesundara, K. J., Matsumoto, K. Anti-hyperglycemic Potential of Natural Products. Mini reviews in medicinal chemistry. 2006,6:349-56.
[123] Bishayee, A., Haznagy-Radnai, E., Mbimba, T., Sipos, P., Morazzoni, P., Darvesh, A. S., et al. Anthocyanin-rich black currant extract suppresses the growth of human hepatocellular carcinoma cells. Natural product communications. 2010,5:1613-8.
[124] Darvesh, A. S., Carroll, R. T., Bishayee, A., Geldenhuys, W. J., Van der Schyf, C. J. Oxidative stress and Alzheimer's disease: dietary polyphenols as potential therapeutic agents. Expert review of neurotherapeutics. 2010,10:729-45.
[125] Wang, Q., Xia, M., Liu, C., Guo, H., Ye, Q., Hu, Y., et al. Cyanidin-3-O-beta-glucoside inhibits iNOS and COX-2 expression by inducing liver X receptor alpha activation in THP-1 macrophages. Life sciences. 2008,83:176-84.
[126] Pergola, C., Rossi, A., Dugo, P., Cuzzocrea, S., Sautebin, L. Inhibition of nitric oxide biosynthesis by anthocyanin fraction of blackberry extract. Nitric Oxide. 2006,15:30-9.
[127] Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T., Mazur, M., Telser, J. Free radicals and antioxidants in normal physiological functions and human disease. The international journal of biochemistry & cell biology. 2007,39:44-84.
[128] Rutkowski, R., Pancewicz, S. A., Rutkowski, K., Rutkowska, J. [Reactive oxygen and nitrogen species in inflammatory process]. Polski merkuriusz lekarski : organ Polskiego Towarzystwa Lekarskiego. 2007,23:131-6.
[129] Franzon, R., Lamers, M. L., Stefanello, F. M., Wannmacher, C. M., Wajner, M., Wyse, A. T. Evidence that oxidative stress is involved in the inhibitory effect of proline on Na(+),K(+)-ATPase activity in synaptic plasma membrane of rat hippocampus. International journal of developmental neuroscience : the official journal of the International Society for Developmental Neuroscience. 2003,21:303-7.
[130] Wyse, A. T., Bavaresco, C. S., Bandinelli, C., Streck, E. L., Franzon, R., Dutra-Filho, C. S., et al. Nitric oxide synthase inhibition by L-NAME prevents the decrease of Na+,K+-ATPase activity in midbrain of rats subjected to arginine administration. Neurochem Res. 2001,26:515-20.
[131] Fedele, E., Ansaldo, M. A., Varnier, G., Raiteri, M. Benzodiazepine-sensitive GABA(A) receptors limit the activity of the NMDA/NO/cyclic GMP pathway: a microdialysis study in the cerebellum of freely moving rats. J Neurochem. 2000,75:782-7.

[132] Paula-Lima, A. C., De Felice, F. G., Brito-Moreira, J., Ferreira, S. T. Activation of GABA(A) receptors by taurine and muscimol blocks the neurotoxicity of beta-amyloid in rat hippocampal and cortical neurons. Neuropharmacology. 2005,49:1140-8.

Table 1. *Structural identification of anthocyanins.*



Anthocyanins	R1	R2	Formula	M.W
Cyanidin	OH	H	C15H11O6	322,72
Malvidin	OCH3	H	C16H13O6	336,74
Delphinidin	OH	OH	C15H11O7	338,72
Petunidin	OCH3	OH	C16H13O7	352,74
Malvidin	OCH3	OCH3	C17H15O7	366,77

Table 2

PCR primers design

Proteins	Primer sequences (50–30)	Accession number (mRNA)
β-Actin[a]	F-TATGCCAACACAGTGCTGTCTGG R-TACTCCTGCTTCCTGATCCACAT	ENSDART00000055194
AChE[a]	F-GACTGCCTTTATCTTAATGTG R-CGGCTGATGAGAGATTCATTG	NP_571921

[a] According to [64]

Table 3

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

Table 4

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 ± SEM for 6-10 animals in each group.

Figure 1

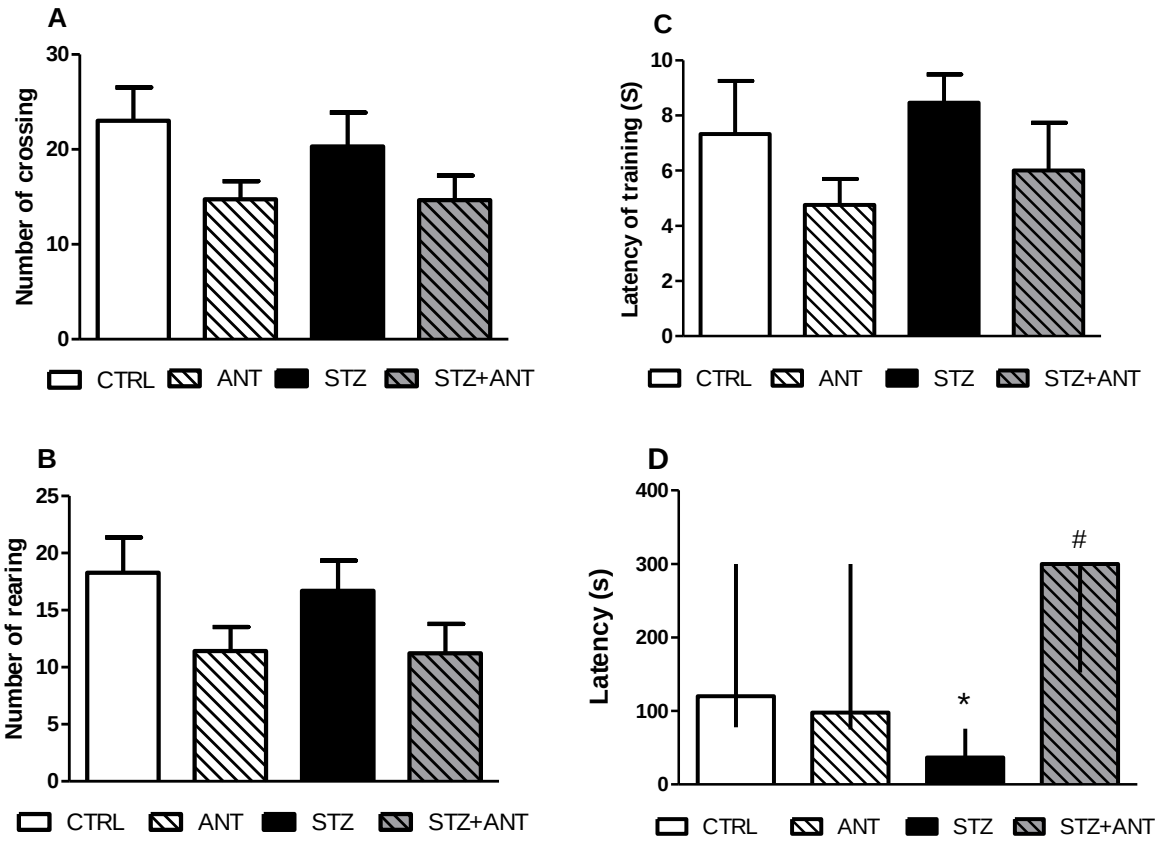

A
Number of crossing
30
20
10
0
CTRL
ANT
STZ
STZ+ANT
C
Latency of training (S)
10
8
6
4
2
0
CTRL
ANT
STZ
STZ+ANT
B
Number of rearing
25
20
15
10
5
0
CTRL
ANT
STZ
STZ+ANT
D
Latency (s)
400
300
200
100
0
#
*
CTRL
ANT
STZ
STZ+ANT

Figure 2

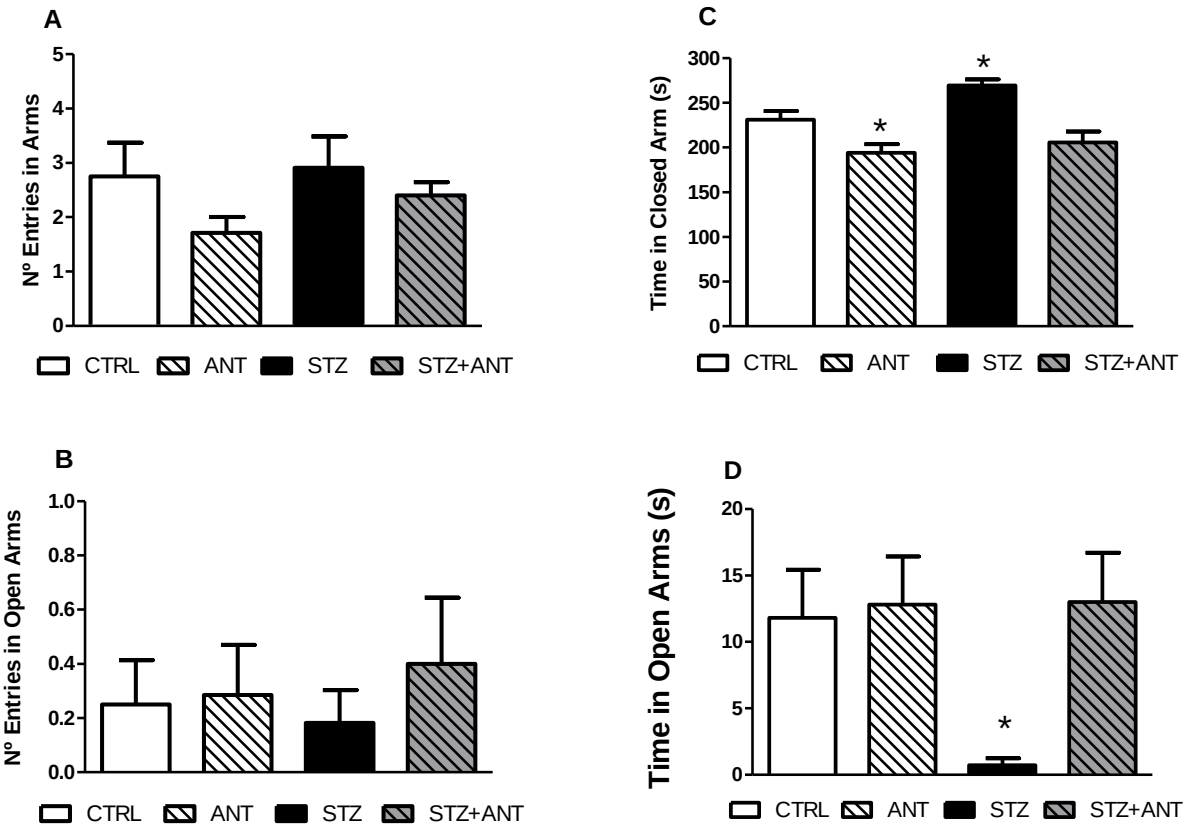

A
Nº Entries in Arms
0
1
2
3
4
5
CTRL
ANT
STZ
STZ+ANT
C
Time in Closed Arm (s)
0
50
100
150
200
250
300
*
*
CTRL
ANT
STZ
STZ+ANT
B
Nº Entries in Open Arms
0.0
0.2
0.4
0.6
0.8
1.0
CTRL
ANT
STZ
STZ+ANT
D
Time in Open Arms (s)
0
5
10
15
20
*
CTRL
ANT
STZ
STZ+ANT

Figure 3

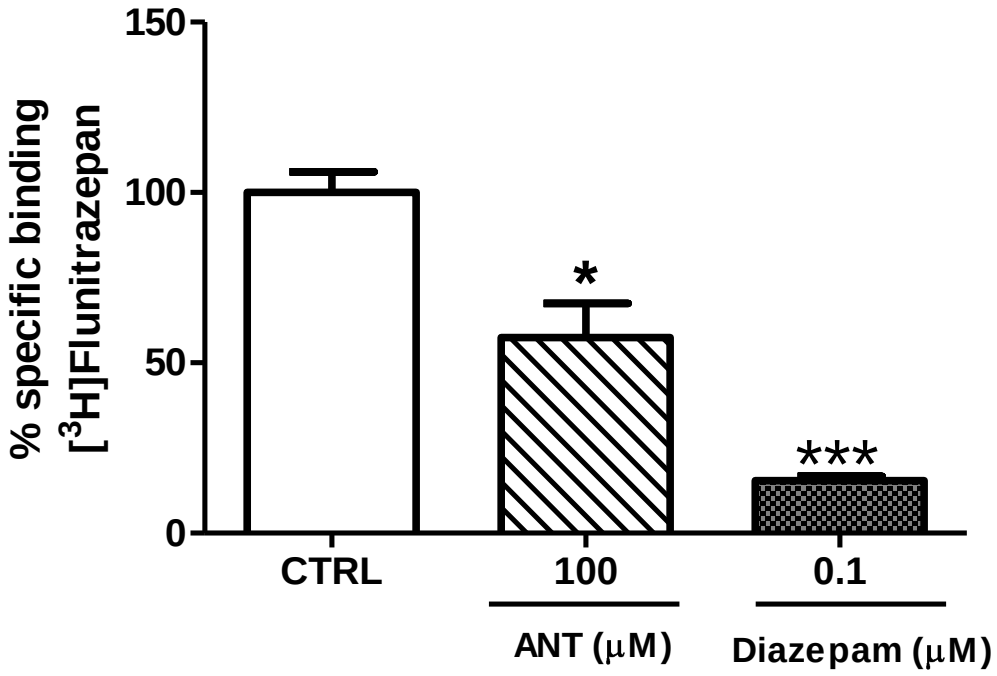

150
100
50
0
% specific binding
[3H]Flunitrazepan
*
***
CTRL
100
0.1
ANT (μM)
Diazepam (μM)

Figure 4

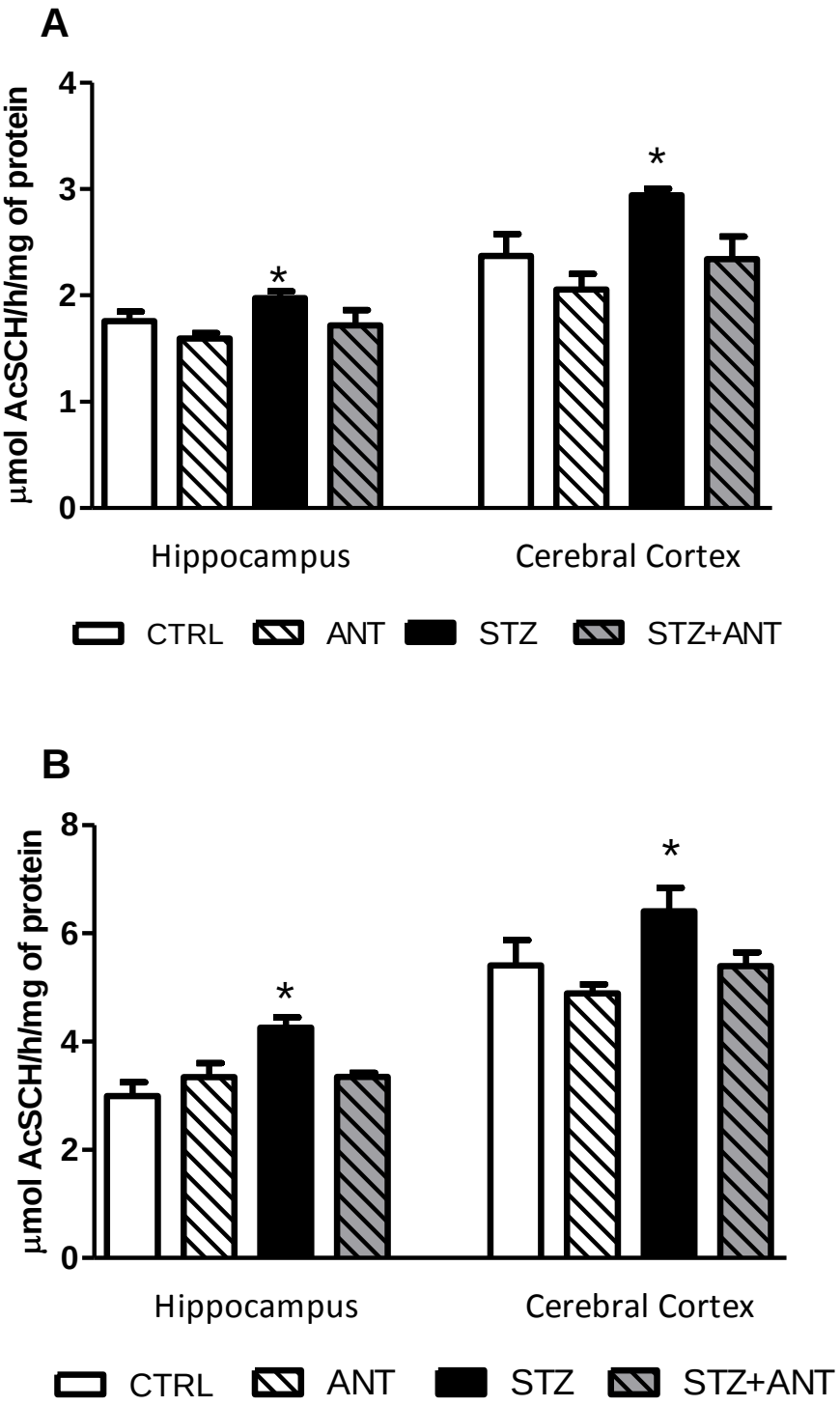

A
µmol AcSCH/h/mg of protein
4
3
2
1
0
*
*
Hippocampus
Cerebral Cortex
CTRL
ANT
STZ
STZ+ANT
B
µmol AcSCH/h/mg of protein
8
6
4
2
0
*
*
Hippocampus
Cerebral Cortex
CTRL
ANT
STZ
STZ+ANT

Figure 5

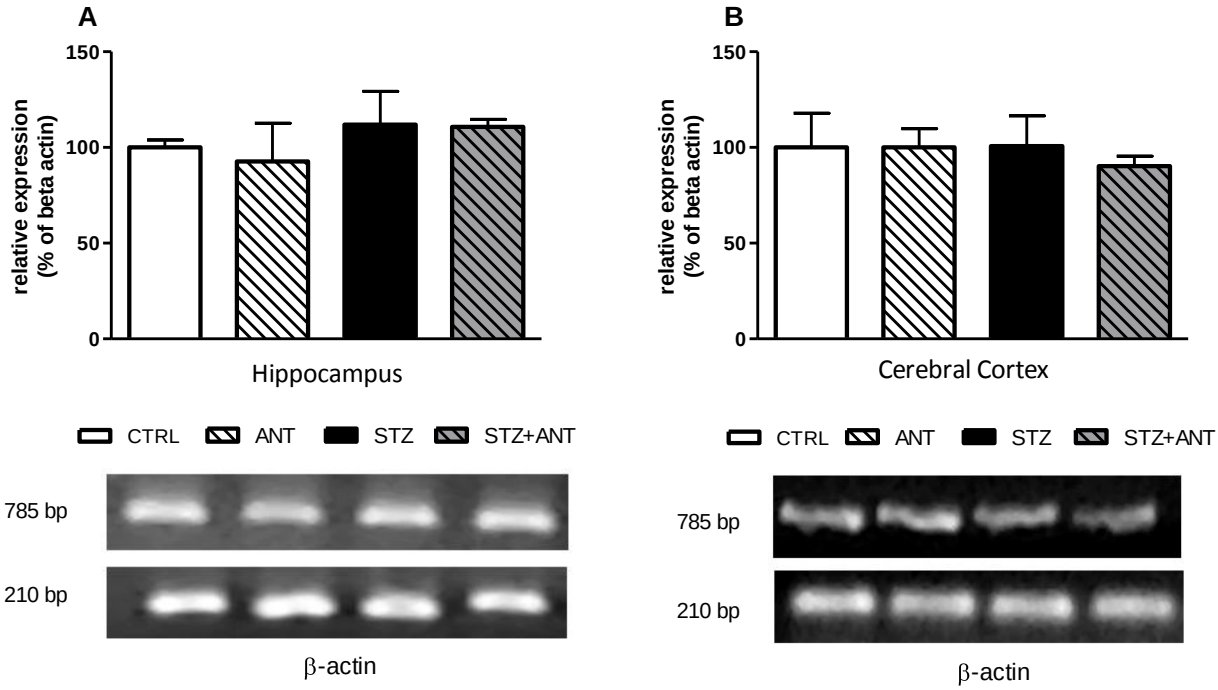

A
150
100
50
0
relative expression
(% of beta actin)
Hippocampus
CTRL
ANT
STZ
STZ+ANT
785 bp
210 bp
β-actin
B
150
100
50
0
relative expression
(% of beta actin)
Cerebral Cortex
CTRL
ANT
STZ
STZ+ANT
785 bp
210 bp
β-actin

Figure 6

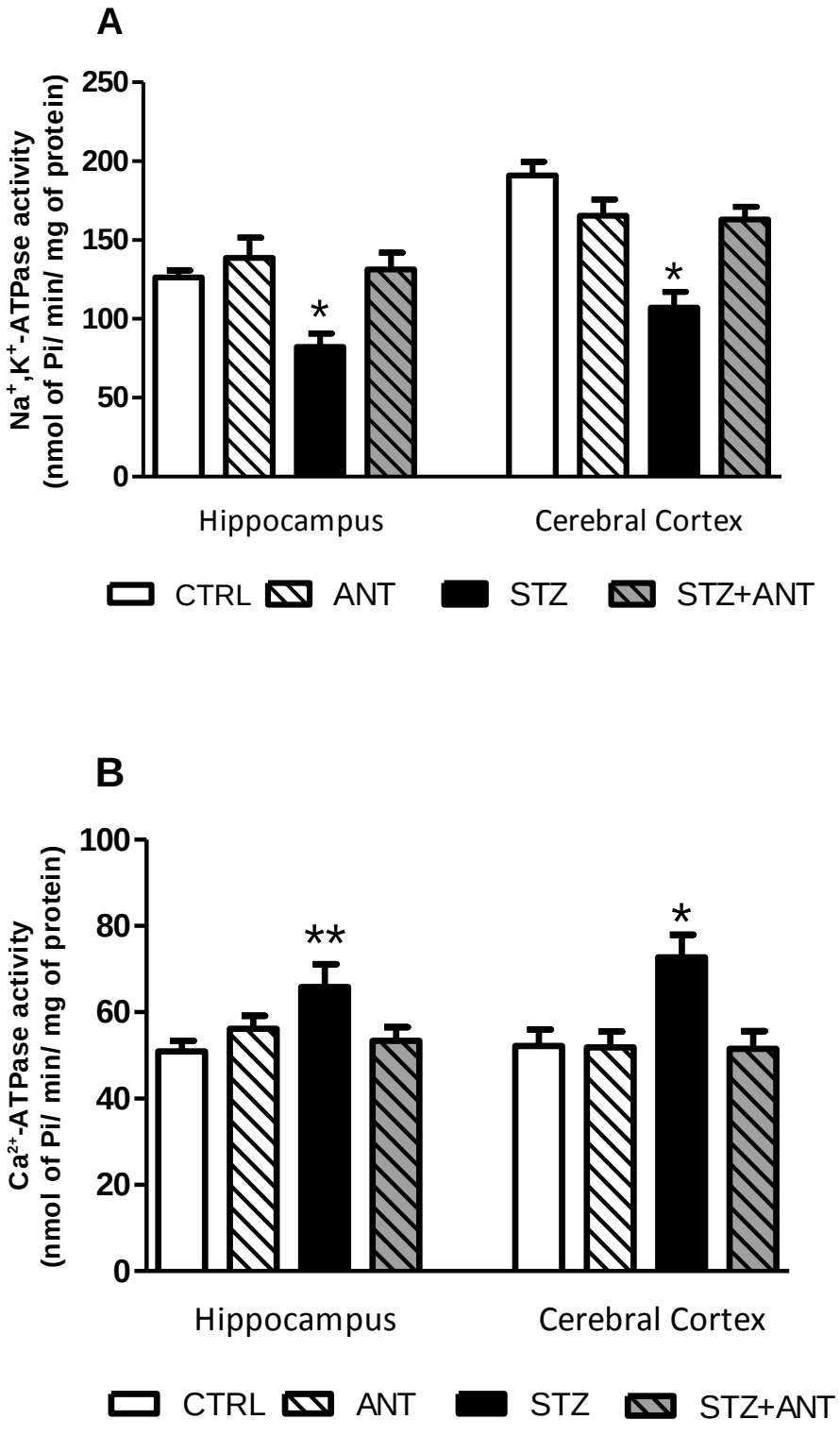
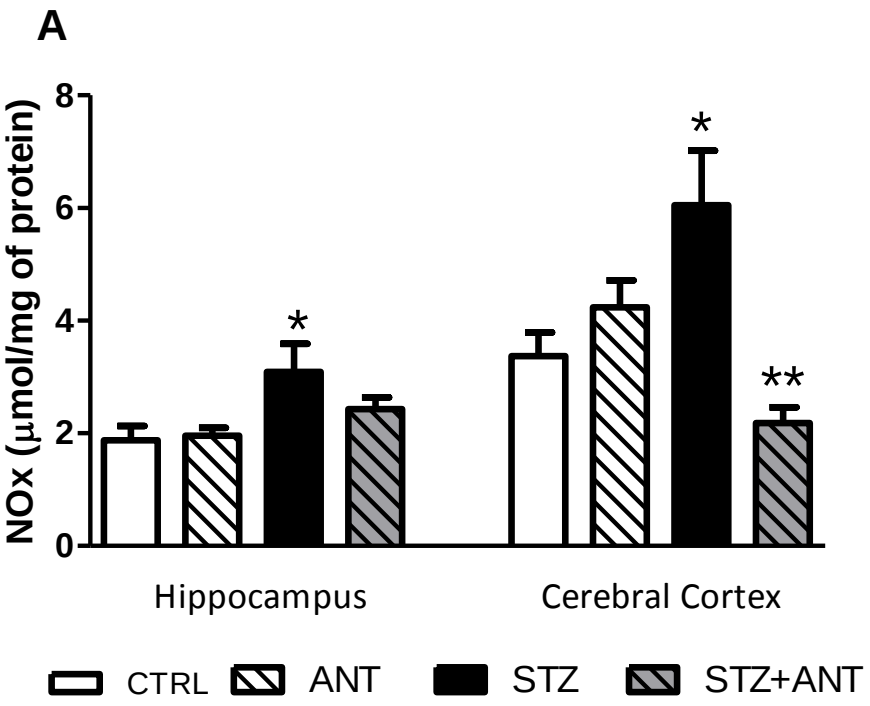

A
Na+,K+-ATPase activity
(nmol of Pi/ min/ mg of protein)
250
200
150
100
50
0
*
*
Hippocampus
Cerebral Cortex
CTRL
ANT
STZ
STZ+ANT
B
Ca2+-ATPase activity
(nmol of Pi/ min/ mg of protein)
100
80
60
40
20
0
**
*
Hippocampus
Cerebral Cortex
CTRL
ANT
STZ
STZ+ANT

Figure 7


A
NOx (µmol/mg of protein)
8
6
4
2
0
*
*
**
Hippocampus
Cerebral Cortex
CTRL
ANT
STZ
STZ+ANT

6. Anexo I

Resultados complementares, em fase de preparação.

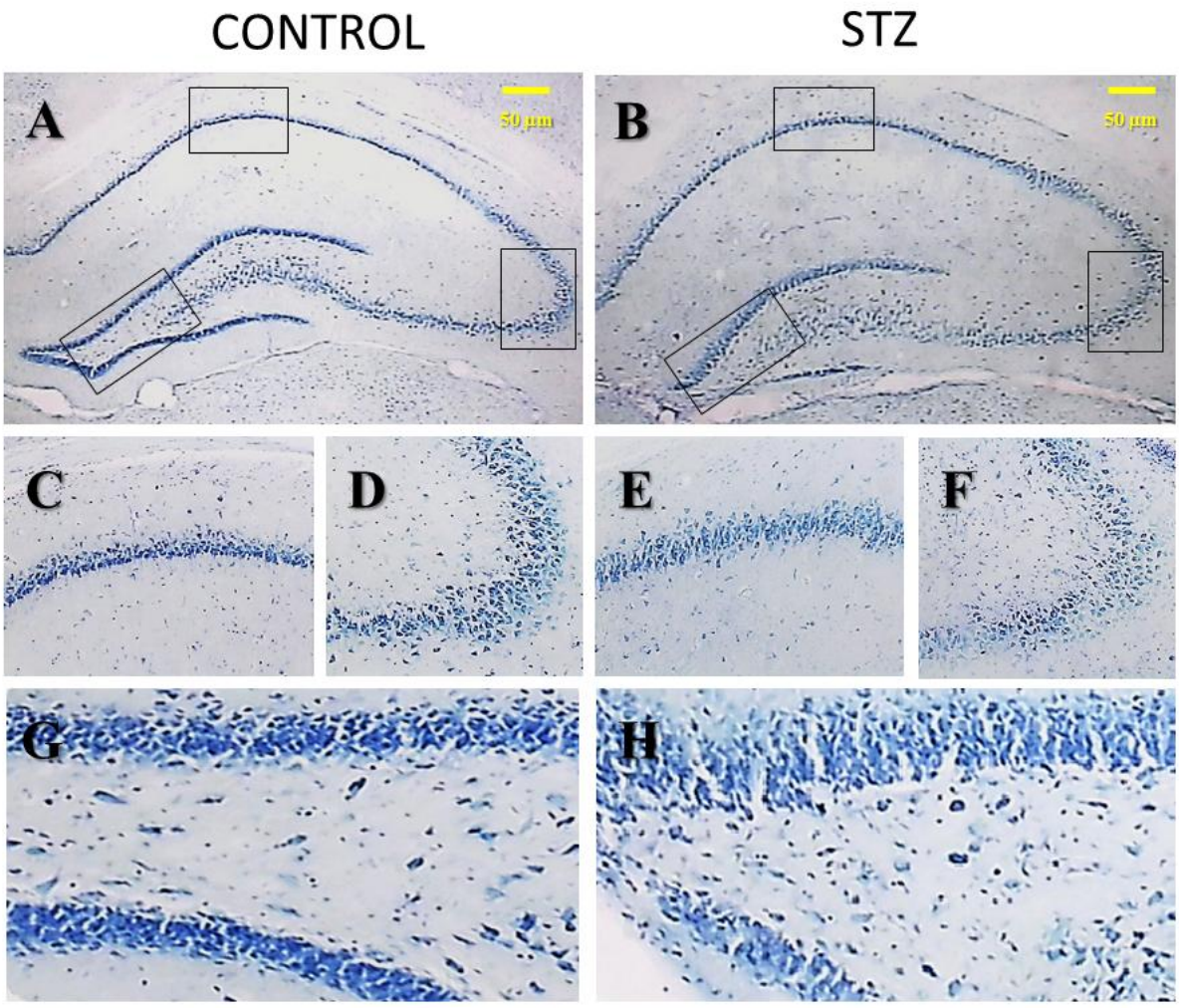


Figure 1. Cresyl violet staining in the hippocampal region of control group (A) and ICV-STZ group (B) at 28 days of treatment. In the ICV-STZ group, only a few neurons in CA1 (E), CA3 (F) region are stained compared to CA1 (C) and CA3 (D) region of control group. The dentate gyrus (G) of control group showed many cresyl violet-positive cells are found in compared to dentate gyrus (H) of ICV STZ group. CA1, cornu ammonis 1; CA3, cornu ammonis 3; dentate gyrus. Scale bar=50 µm.

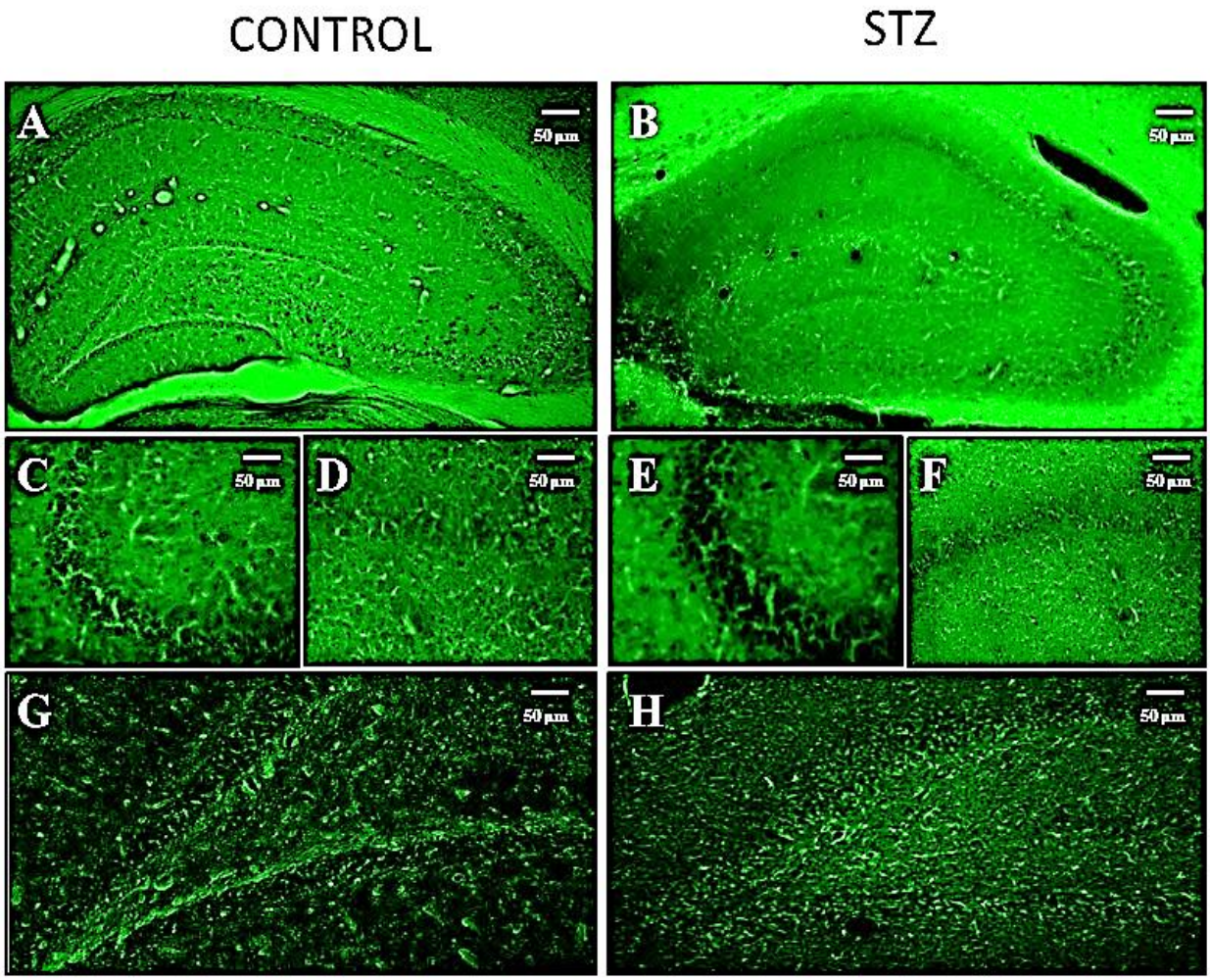


Figure 2 Fluoro-Jade C histochemistry staining in hippocampus (CA1, CA3 and giro dentate) after 28 days of the intracerebroventricular STZ or vehicle injections. Digital images of rat brain sections showing no difference for Fluoro-Jade C-positive neurons in hippocampus regions of control (CA1, CA3 and gyro dentate) or ICV-STZ injections (CA1, CA3 and gyro dentate). Original magnification: (A) and (B)×5, (C-H)x20; Scale bar=50 µm.

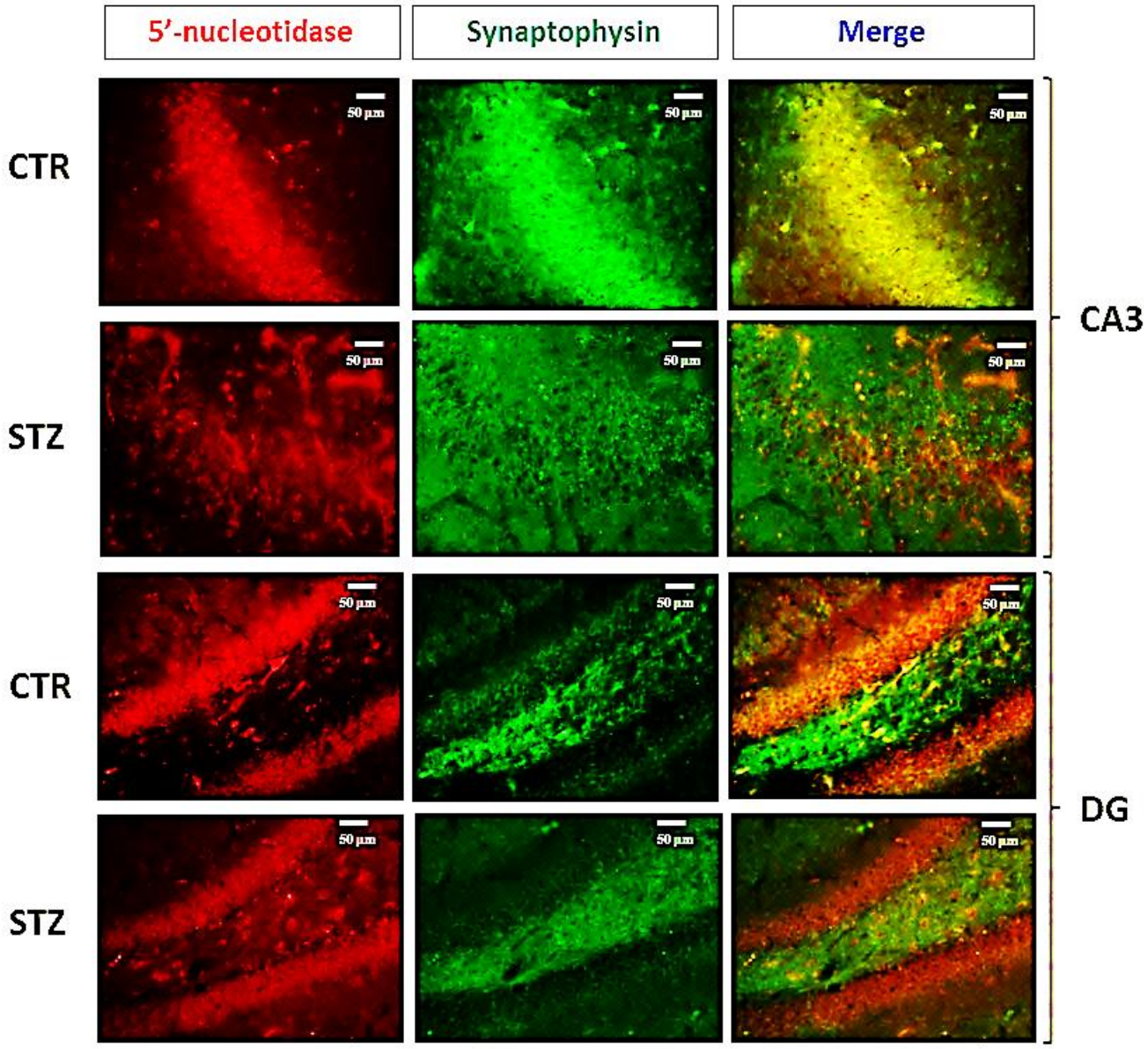


Figure 3. Fluorescence Immunohistochemistry of 5'-nucleotidase (red) and synaptophysin (green) in ICV-STZ and normal brains. Examples of coronal sections of the hippocampus from ICV-STZ and normal brains. 5'-nucleotidase (5'-NT) immunoreactivity in CA3 and dentate gyrus (DG) were observed in normal rat brains, but ICV-STZ group showed lower reactivity for 5'-NT both in CA3 or DG. Similarly, ICV-STZ injection showed weaker immunoreactivity for synaptophysin in CA3 and dentate gyrus compared to the normal brains. Original magnification: (A)×20.

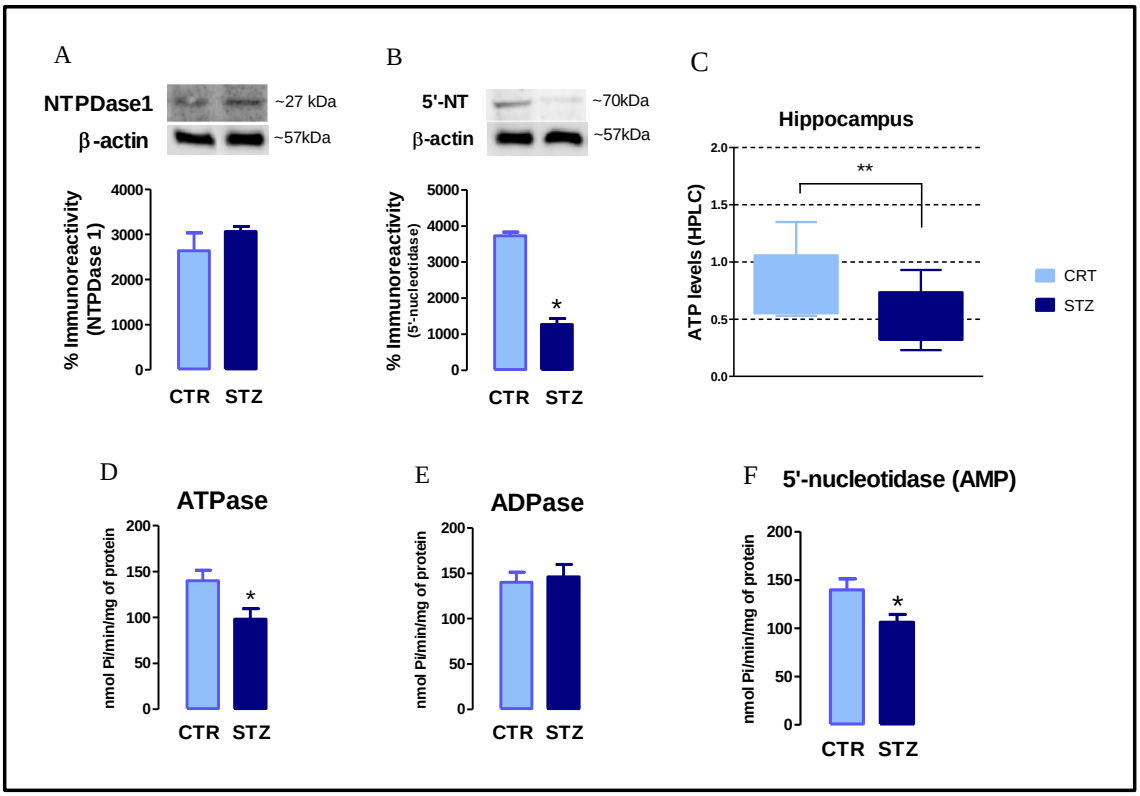


Figure 4 - Intracerebroventricular injection of STZ alter proteins density for NTPDase 1 (A) and 5'-NT (B) were determined by Western-blot analysis. Equalization of protein loading was determined using β-actin as the house keeping protein. (C) ATP levels measured by high pressure liquid chromatography (HPLC). (D) ATPase activity; (E) ADPase activity and (F) 5'NT activity in synaptosomes of hippocampus of CTR and ICV-STZ groups. Data (mean values±SEM) were submitted to t-test statistical analysis. Significance level adopted was **P*<0.05 versus CTR group.

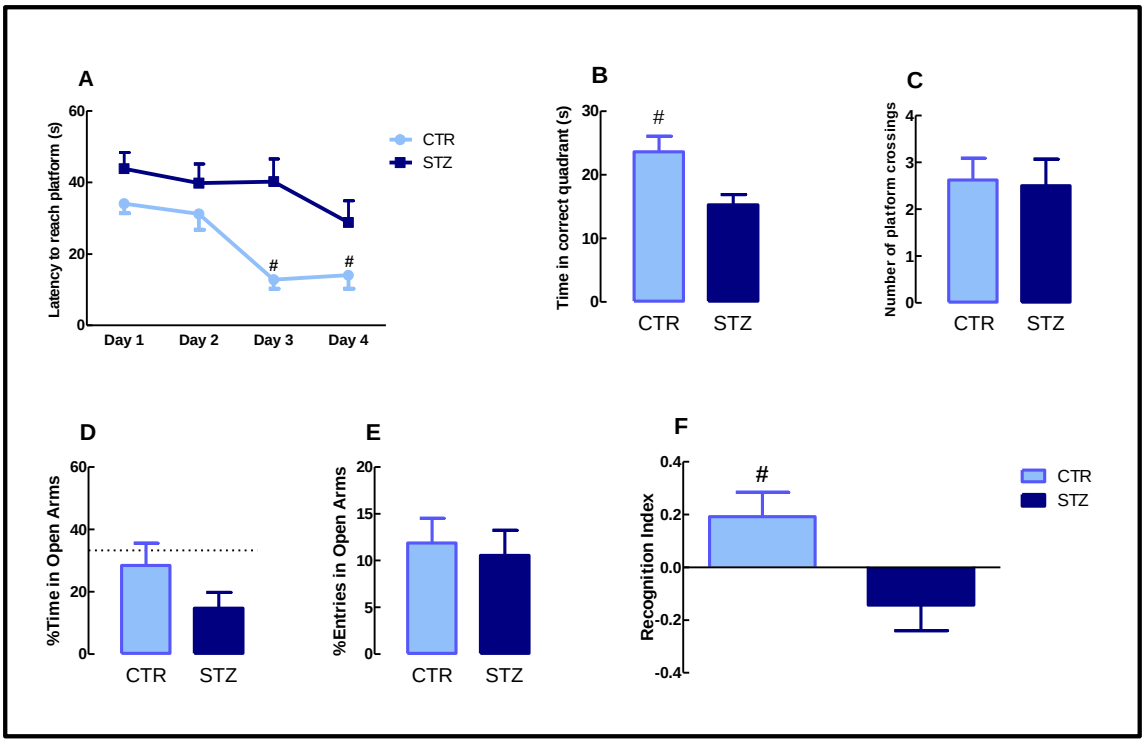


Figure 5. Intracerebroventricular injection of STZ display changes in cognitive behavior. (A) The CTR groups significantly differ in search times during acquisition training. (B) Probe test after the original training. (C) Number of crossings on the platform. (D) panels plot the % relative time and (E) % of entries in open arms in Elevated plus maze task during the 5-minutes. (F) Novel object recognition assessment of CTR and STZ groups. Recognition index = (time spent exploring a novel object) / (total time spent exploring both novel and familiar objects). # $p<0.05$; One sample t test relative to CTR (n = 10).

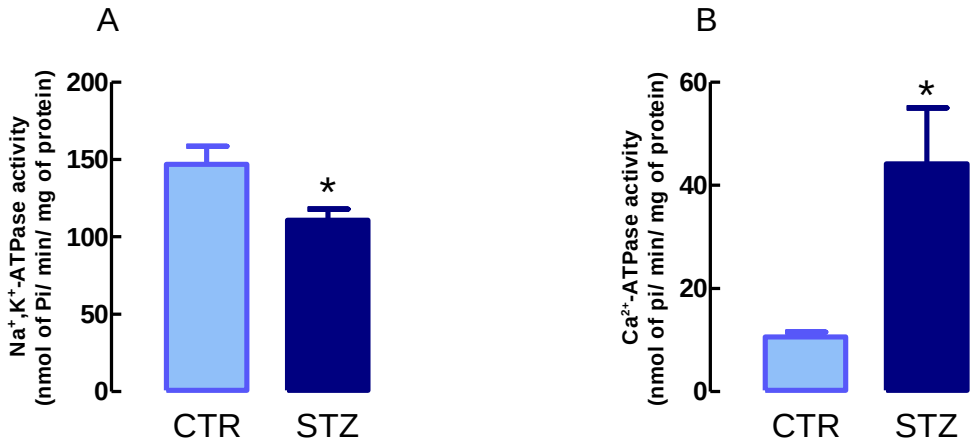


Figure 6. Na^+,K^+-ATPase (A) and Ca^+-ATPase (B) activity in hippocampus of intracerebroventricular STZ or vehicle injections. Data (mean values±SEM) were submitted to t-test statistical analysis. Significance level adopted was $P<0.05$.

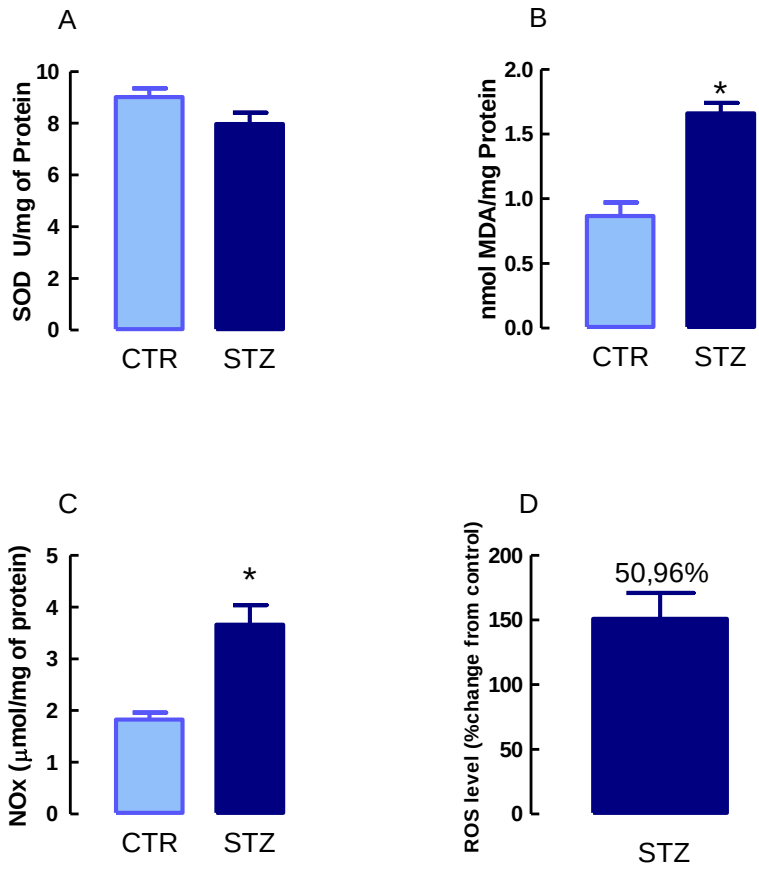


Figure 7. Effects of intracerebroventricular STZ or vehicle injection on superoxide dismutase activity, MDA, nitrite plus nitrate levels and ROS generation of the hippocampus. Graphs

Data (mean values±SEM) were submitted to t-test statistical analysis, (D) Graph express changes in ROS generation (percentage) of STZ group in relation to CTR group. Significance level adopted was *P*<0.05.

Table 1 – Primary and secondary antibodies for immunohistochemistry

Antibody	Supplier	Host	Type	Dilution
Synaptophysin	Millipore	Mouse	Monoclonal	1:200
5'-nucleotidase	Sevigny Lab	Rabbit	Monoclonal	1:500
Anti-mouse Alexa-Fluor 488	Invitrogen	Donkey	IgG (H+L)	1:400
Anti-rabbit Alexa-Fluor 594	Invitrogen	Donkey	IgG (H+L)	1:400

Primary antibodies for Western Blot analysis

Antibody	Supplier	Host	Type	Dilution
NTPDase1	Sevigny Lab	Rabbit	Polyclonal	1:1000
5'-nucleotidase	Santa Cruz	Mouse	Polyclonal	1:1000
β-actin	Sigma	Mouse	Monoclonal	1:20000

Table 2 – Reagents used and respective suplier

Reagent	Supplier
Sodium thiopental	B.Braun Medical (Portugal)
Sucrose	Sigma-Aldrich (Portugal)
Tissue-Tek	(Sakura-Americas, USA)
TMED	Sigma-Aldrich (Portugal)
Triton - x -100	Sigma-Aldrich (Portugal)
Trizma base	Sigma-Aldrich (Portugal)
Tween	Sigma-Aldrich (Portugal)

Table 3 – Reagents used and respective suplier

Reagent	Supplier
30% Acrylamide/Bis solution	Bio Rad (Portugal)
Ammonium persulfate (APS)	Sigma-Aldrich (Portugal)
Bicine	Sigma-Aldrich (Portugal)
BCA Kit	Thermo scientific (USA)
Bovine serum albumin (BSA)	Sigma-Aldrich (Portugal)
Calcium chloride (CaCl2)	Sigma-Aldrich (Portugal)
CAPS ([3-(cyclohexylamino)-1-propane-sulfonic acid)	Sigma-Aldrich (Portugal)
Citric acid	Sigma-Aldrich (Portugal)
CLAP (cocktail of proteases inhibitors)	Sigma-Aldrich (Portugal)
DAKO Fluorescence Mounting Medium	DAKO (Denmark)
ECF	GE Healthcare (United Kingdom)
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich (Portugal)
Gelatin	Sigma-Aldrich (Portugal)
Glucose	Sigma-Aldrich (Portugal)
Glycerol	Sigma-Aldrich (Portugal)
Halothane	Sigma-Aldrich (Portugal)
HEPES	Sigma-Aldrich (Portugal)
Hydrochloric acid (HCl)	Sigma-Aldrich (Portugal)
Magnesium Chloride (MgCl)	Sigma-Aldrich (Portugal)
Metanol	Sigma-Aldrich (Portugal)
Normal Horse Serum (NHS)	Invitrogen (United Kingdom)
Paraformaldehyde	Sigma-Aldrich (Portugal)
Percoll	GE Healthcare (United Kingdom)
Penylmethanesulfonylfluoride (PMSF)	Sigma-Aldrich (Portugal)
Potassium chloride (KCl)	Sigma-Aldrich (Portugal)
Sodium dodecyl sulfate (SDS)	Bio Rad (Portugal)
Sodium azide	Sigma-Aldrich (Portugal)
Sodium Bicarbonate (NaHCO3)	Sigma-Aldrich (Portugal)
Sodium Chloride (NaCl)	Sigma-Aldrich (Portugal)
Sodium phosphate monobasic (NaH2PO4)	Sigma-Aldrich (Portugal)

7. Discussão

7.1 Discussão

O envelhecimento tem sido reconhecido como um processo irreversível e inevitável desde os tempos antigos. Distúrbios associados ao envelhecimento incluem disfunção imunológica (Candore, Balistreri *et al.*, 2006), degeneração cognitiva (Barzilai, Atzmon *et al.*, 2006), doença cardiovascular (Dominguez e Barbagallo, 2007) e síndrome metabólica (Maggi, Noale *et al.*, 2008). Diversas evidências sugerem que o envelhecimento aumenta o risco de degeneração do sistema nervoso, afetando principalmente a retenção de memória, gerando perda de memória e disfunções cognitivas em idosos (Shih, Chan *et al.*, 2010).

Estudos tem demonstrado que as antocianinas são capazes de melhorar a memória de ratos velhos no labirinto aquático de Morris (Andres-Lacueva, Shukitt-Hale *et al.*, 2005), na tarefa da esquiva inibitória em ratos (Barros, Amaral *et al.*, 2006) e também em seres humanos idosos (Krikorian, Shidler *et al.*, 2010; Krikorian, Boespflug *et al.*, 2012). Alguns investigadores descobriram que os flavonóides, incluindo algumas antocianinas, possuem uma biodisponibilidade oral em ratos (Mcghie, Ainge *et al.*, 2003) e que são capazes de atravessar a barreira hemato encefálica após suplementação com mirtilo (Andres-Lacueva, Shukitt-Hale *et al.*, 2005) e amora (Talavera, Felgines *et al.*, 2003; Talavera, Felgines *et al.*, 2004; Talavera, Felgines *et al.*, 2005), bem como depois de uma única administração (Passamonti, Vrhovsek *et al.*, 2005), sugerindo que estes compostos possam ter um efeito direto sobre os processos cerebrais. Adicionalmente, o consumo alimentar para indivíduos foi estimada ser de até 200 mg/dia de antocianinas, que é maior do que a de outros flavonóides como a quercitina (23 mg/dia) (Scalbert e Williamson, 2000; Mcghie, Ainge *et al.*, 2003). Nesta tese, nosso objetivo inicial foi investigar o papel das antocianinas sobre a memória e relacionar estas mudanças com o sistema colínergico e purinérgico em um modelo de amnésia induzido por scopolamina. Uma vez encontrado efeitos deste composto sobre parâmetros comportamentais, especialmente memória, definimos o passo seguinte, que seria investigar se o tratamento com antocianinas poderia prevenir déficits encontrados em um modelo de demência esporádica do tipo Alzheimer, induzido por administração intracerebroventricular de streptozotocina (ICV-STZ), comparar os resultados nos dois modelos e sugerir que tanto o sistema colinérgico como

purinérgico podem ser regulados pelo tratamento com antocianinas, sugerindo assim, o uso deste composto como uma nova estratégia terapêutica para controlar a deteriorização mnemônica no envelhecimento.

Nosso primeiro estudo (Manuscrito I) foi conduzido para avaliar o papel preventivo das antocianinas (ANT) nos déficits de memória induzidos por escopolamina (SCO) em ratos. Nossos resultados mostraram que a SCO prejudicou a aquisição de memória em ratos treinados na tarefa de esquiva inibitória. Curiosamente, neste estudo verificou-se que uma dose de 200 mg/kg de ANT durante sete dias não melhorou *per se* a memória de ratos na tarefa de esquiva inibitória, mas impediu que os déficits de memória induzidos pela SCO ocorre-se. A SCO é um alcalóide derivado de *Atropa belladonna* e atua como um antagonista competitivo do receptor de acetilcolina muscarínico M1 (mAChR) (Wang, Lu *et al.*, 2003). Por esta razão, a SCO é usada para comprometer a neurotransmissão colinérgica e, em alguns aspectos, reproduz o déficit de memória observado em doenças caracterizadas por uma deficiência na neurotransmissão colinérgica, tais como a DA (Christensen, Maltby *et al.*, 1992). E neste primeiro estudo, os resultados mostraram que a administração de SCO aumentou significativamente a atividade de acetilcolinesterase (AChE), no córtex cerebral e no hipocampo de animais, e estes resultados são consistentes com o de outros autores (Choi, Lee *et al.*, 2012; Oh, Kim *et al.*, 2013). A SCO tem sido utilizada para investigar drogas antiamnésicas para o SNC relacionado com a idade (Sakurai, Kato *et al.*, 1998). Em investigações *in vivo* de ratos tratados com SCO, foi mostrado que a AChE cerebral foi marcadamente reduzida (Southam, Thomas *et al.*, 1991). Nossos resultados demonstraram que SCO aumentou a atividade da AChE e este efeito foi prevenido pelo tratamento com ANT. Estes dados, juntamente com os parâmetros de memória sugerem que as ANT pode exercer seus mecanismos pela regulação da via colinérgica.

Além das alterações na transmissão colinérgica, distúrbios cognitivos, apresentam uma diminuição da geração do potencial de membrana e o influxo de Ca^{2+} neuronal (Mata, Berrocal *et al.*, 2011). Considerando que a Na^{+}, K^{+}-ATPase é uma das mais abundantes enzimas do SNC, consumindo cerca de 40-60% do ATP gerado (Kaplan, 2002), não é surpreendente que as alterações na sua atividade podem causar uma variedade de anormalidades. Neste primeiro estudo, foi encontrado uma redução na atividade de Na^{+}, K^{+}-ATPase e Ca^{2+}-ATPase no córtex cerebral e no hipocampo de animais tratados com SCO, o qual foram atenuadas

pelo tratamento com ANT (200mg/kg). Adicionalmente, a atividade reduzida da Na^+, K^+-ATPase e da Ca^{2+}-ATPase, tem sido sugerido desempenhar um papel central no processo de formação da memória (Lingrel, Williams *et al.*, 2007; Moseley, Williams *et al.*, 2007) e na patogénese de doenças neurodegenerativas, tais como a DA (Mata, Berrocal *et al.*, 2011) e doença de Parkinson (Zaidi, 2010).

No SNC, as NTPDases são importantes enzimas envolvidas na neurotransmissão purinérgica. E no segundo estudo realizado por nosso grupo de pesquisa (ARTIGO 1), foi observado um aumento significativo na atividade das NTPDases em sinaptossomas de cortex cerebral e hipocampo em ratos tratados com SCO, e este efeito pode ser responsável pela redução do ATP extracelular na fenda sináptica, ao qual comprometeria a sinalização purinérgica, uma vez que reduz a disponibilidade de ATP extracelular. Além disso, estas enzimas diferem nas suas proporções e preferência para substrato. O NTPDases 1, hidrolisa ATP em ADP e da mesma forma, também NTPDases 2, 3 e 8, hidrolisam mais ATP do que ADP, e, preferencialmente, a NTPDase 4 hidrólisa UDP (Zimmermann, 2001). Com base nestes resultados, sugere-se que os efeitos encontrados no hipocampo e no córtex cerebral podem estar relacionados com a atividade das diferentes isoformas da enzima. A hidrólise enzimática de ATP e ADP no hipocampo sugere o envolvimento da NTPDases 1, ao passo que no córtex cerebral outras isoformas, com maior afinidade para o ATP podem estar envolvidas. Estes resultados parecem fazer algum sentido quando comparados com os resultados de memória avaliados na tarefa de esquiva inibitória, onde o efeito amnésico induzido pela SCO foi impedido pelo tratamento com ANT. Avaliou-se também, neste modelo de amnésia, a atividade da adenosina deaminase (ADA) e 5'-nucleotidase (5'-NT). Como pode ser visto no Artigo 1, a SCO diminuiu a atividade da ADA e da 5'-NT em sinaptossomas de hipocampo, e o tratamento com ANT foi capaz de prevenir esta diminuição. Em adição, a atividade da 5'-NT é muito importante uma vez que leva à produção de adenosina extracelular (Schetinger, Vieira *et al.*, 2001; Burnstock, 2006).

A depleção da adenosina extracelular pode perturbar a formação da memória, pois a adenosina tem sido considerada um neuromodulador importante no estabelecimento da LTP e LTD, bem como na plasticidade sináptica (De Mendonca, Costenla *et al.*, 2002). Com base na atividade das ectonucleotidases, pode ser visto que o tratamento com SCO aumentou a hidrólise de ATP e de adenosina

extracelular. A redução dos níveis extracelulares dessas moléculas pode reduzir a sinalização da neurotransmissão adenosinérgico e purinérgica, respectivamente, e comprometer a aquisição da formação da memória. No entanto, nenhum estudo na literatura mostra o efeito da SCO e do tratamento com ANT sobre a atividade de ectonucleotidases, devido ao fato da escasses de trabalhos que investiguem estas abordagens, o possível mecanismo pelo qual a SCO ou as ANT atuam no sistema coliérgico e purinérgico precisam ser ainda investigados.

Uma vez que um grande número de evidências sugere um papel neuroprotetor para ANT, e que os primeiros objetivos desta tese foram alcançados, em seguida foi investigado no segundo manuscrito desta tese (Manuscrito II) se este composto natural é capaz de prevenir as alterações encontradas num modelo de demência esporádica do tipo de Alzheimer (DAS), induzida por injecção ICV-STZ. A administração intracerebroventricular de STZ (3mg/kg), em uma dose subdiabetogênica para roedores têm sido relatada como apropriado modelo para a DAS (De La Monte, Tong *et al.*, 2006) causando um enfraquecimento progressivo da memória, perda sináptica e disfunção neuronal (Blokland e Jolles, 1993). Os resultados obtidos mostraram que o ICV-STZ prejudica a aquisição de memória em ratos treinados na tarefa de esquiva inibitória, e nesse estudo utilizou-se também a dose de 200 mg/kg de ANT, mostrando que este composto foi capaz de impedir os déficits de memória encontrados em ratos tratados com ICV-STZ. Além disso, nos objetivos iniciais desta tese, definimos que a dose 200mg/kg de ANT antagonizou os déficits de desempenho mnemônico induzidos por SCO em ratos, o que sugere que as ANT possuem uma estreita interação com o sistema colinérgico. A ingestão diária de ANT foi escolhida por estar de acordo com o consumo da população dos Estados Unidos (Manach, Scalbert *et al.*, 2004). Em consonância, estudos anteriores indicam também que uma ingestão diária de 200 mg/kg de ANT apresentam efeitos protetores em ratos, camundongos e humanos (Blokland e Jolles, 1993) e pode melhorar a aprendizagem e memória em ratas velhas e ovarioectomizadas (Varadinova, Docheva-Drenska *et al.*, 2009).

Além da aprendizagem e avaliação de memória, neste segundo manuscrito, avaliamos o comportamento de ansiedade dos animais pela tarefa de labirinto em cruz elevado (Belzung e Griebel, 2001). Os nossos resultados mostraram uma ansiedade maior no grupo ICV-STZ (3 mg/kg), que está de acordo com observações anteriores em que os ratos submetidos a ICV-STZ, em curto período de tempo (7

dias) de tratamento apresentam um aumento no comportamento ansioso (Pinton, Da Rocha *et al.*, 2011). Porém, também testou-se um protocolo de exposição maior ao ICV-STZ (28 dias, dados preliminares), e os resultados demonstraram que nesse período o comportamento ansiogênico destes animais parece estabilizar (ANEXO I, Figura 5 D e E). Embora não tenha sido encontrado um efeito ansiolítico do tratamento com ANT *per se* por 7 dias para o tempo nos braços abertos, foi encontrado uma diminuição do tempo gasto nos braços fechados pelos animais tratados com ANT. Porém, neste estudo foi utilizada uma dose de ANT que apresenta efeitos benéficos sobre a aprendizagem e memória (Varadinova, Docheva-Drenska *et al.*, 2009; Gutierres, J. M., Carvalho, F. B. *et al.*, 2012). No entanto, sugerimos que para investigar os efeitos ansiolíticos das antocianinas, com maior adquação, seria pertinente selecionar um intervalo de doses. Além disso, a dose escolhida neste estudo foi capaz de prevenir o comportamento ansiogênico causado pela administração ICV-STZ. Por esta razão, sugere-se que o mecanismo pelo qual as ANT desempenham seus efeitos ansiolíticos está relacionado com a afinidade deste composto pelo receptores $GABA_A$, uma vez que neste estudo mostramos que as ANT deslocam significativamente a ligação específica do [^{3}H]flunitrazepam com o sitio benzodiazepínico conhecido deste receptor. E este trabalho é o primeiro a descrever um local específico onde este composto pode agir de forma a promover um efeito ansiolítico, sugerindo que as ANT podem ser considerados como um agente farmacológico em situações de ansiedade.

O papel fundamental do sistema colinérgico na memória é ainda sublinhado pelo uso de inibidores da acetilcolinesterase na DA, com o intuito de evitar o declínio da memória. Neste estudo, verificou-se ainda, que o grupo ICV-STZ apresentou um aumento na atividade da AChE em sobrenadante e sinaptosomas de hipocampo e córtex cerebral em relação a todos os grupos testados. Este resultado está em conformidade com os estudos anteriores que mostram aumento na expressão de AChE (Lester-Coll, Rivera *et al.*, 2006) e de sua atividade com a administração intracerebroventricular de STZ (Awasthi, Tota *et al.*, 2010)[. Além disso, a injeção ICV-STZ também causou deficiência colinérgica apoiada pela redução da atividade cholineacetiltransferase (CHAT) no hipocampo de ratos (Blokland e Jolles, 1993; Sonkusare, Srinivasan *et al.*, 2005). É possível que o aumento da atividade da AChE ocorra devido a uma diminuição no metabolismo energético e estresse oxidativo levando a disfunções cognitivas, inibindo a síntese de ATP e acetil-CoA. Além disso,

a deficiência na sinalização da insulina e aumento do estresse oxidativo induzido por administração ICV-STZ foi associada com uma regulação positiva de AChE no cérebro de ratos (Lester-Coll, Rivera *et al.*, 2006). O nosso grupo de pesquisa demonstrou que a administração intraperitoneal de STZ (em doses superiores: entre 60-76mg/kg), um modelo de diabetes do tipo 2; provocou um aumento significativo na atividade da AChE em sinaptosomas de córtex cerebral de ratos (Schmatz, Schetinger *et al.*, 2009). Adicionalmente, neste terceiro estudo, foi observado que as ANT foram capazes de prevenir a *up*-regulação de AChE no hipocampo e no córtex cerebral de animais injetados ICV-STZ, mesmo que este composto *per se* não apresenta qualquer efeito sobre a atividade de AChE.

Embora não avaliamos neste estudo se a Na^+, K^+-ATPase ou a Ca^{2+}-ATPase neuronal e glial são preferencialmente afetadas pela administração de ICV-STZ, é concebível que a injeção ICV-STZ pode alterar ambos os tipos de células. Se for este o caso, a administração ICV-STZ poderia alterar os gradientes intracelulares como o Na^+, K^+ e Ca^{2+}, facilitando a despolarização neuronal e prejudicando os processos de transporte dependentes do gradiente de potássio, tais como a captação de neurotransmissores (Benarroch, 2011). Neste ponto de vista, sabe-se que uma diminuição da atividade e expressão de Na^+, K^+-ATPase, afeta diretamente a sinalização de neurotransmissores, prejudicando a aprendizagem e a memória, bem como a atividade locomotora e os comportamentos de ansiedade de ratos (Dos Reis, De Oliveira *et al.*, 2002). E em estudos *in vitro* foi mostrado que o inibidor da Na^+, K^+-ATPase, a ouabaína, aumenta o influxo de Ca^{2+} em fatias de cérebro de ratos (Fujisawa, Kajikawa *et al.*, 1965), induz a liberação de glutamato por transporte reverso de Na^+ [108] e causa a excitotoxicidade em neurônios do hipocampo (Lees, Lehmann *et al.*, 1990). Corroborando com estes achados, o nosso estudo mostrou que a administração ICV-STZ tanto em um curto período (manuscrito II) quando em longo período (ANEXO I, Figura 6 A e B) reduziu a atividade da Na^+, K^+-ATPase e causou um aumento da Ca^{2+}-ATPase, sugerindo que uma perturbação nas concentrações eletrolíticas de Na^+ e de Ca^{2+} pode levar a excitotoxicidade neuronal em um modelo experimental para a DAS.

Além disso, é sabido que a inibição de Na^+, K^+-ATPase pode aumentar as correntes mediadas por receptores NMDA no hipocampo (Zhang, Guo *et al.*, 2012) Sabe-se que a ativação do receptor de NMDA aumenta a produção de óxido nítrico (NO), aumentando tanto a síntese, como a atividade da óxido nítrico sintase (NOS)

(Prast e Philippu, 2001). O NO é um mensageiro retrógrado que se difunde através das membranas celulares e pode ativar a guanilato ciclase e a PKG (East e Garthwaite, 1991). Estudos anteriores têm demonstrado que a ativação da NOS e a síntese de NO está relacionado com uma redução da Na^+, K^+-ATPase (Boldyrev, Bulygina *et al.*, 2004). Os resultados obtidos demonstraram que a administração ICV-STZ, aumenta os níveis de nitrato/nitrito (NOx), que são considerados marcadores da síntese de NO, e estes resultados podem estar relacionados com a redução de Na^+, K^+-ATPase, de duas maneiras: i) O NO pode inibir a Na^+, K^+-ATPase através da sua ligação a grupos tiós, gerando S-nitro-tióis e, consequentemente, conduzindo à formação de compostos nitrosos (Boldyrev e Bulygina, 1997; Boldyrev, Bulygina *et al.*, 1997)[, ii) A ativação da via de sinalização relacionada com a NOS/cGMP/PKG (Carvalho, Mello *et al.*, 2012).

Evidências têm demonstrado uma correlação entre o consumo de frutas e vegetais frescos, com a prevenção e atraso do aparecimento de doenças crônico-degenerativas, incluindo câncer (Juranic e Zizak, 2005) e DA (Darvesh, Carroll *et al.*, 2010). Estes estudos indicaram que, além dos efeitos antioxidantes das ANT, por exemplo, estes compostos podem diminuir os níveis de NO (Blokland e Jolles, 1993). Os dados apresentados neste trabalho demonstram que o tratamento com ANT (200 mg/kg) impediu a elevação no níveis de NOx induzido pela administração ICV-STZ. Estudos anteriores demonstraram que as ANT são capazes de reduzir a expressão de iNOS, bem como a produção de NO em macrófagos e células JC77 expostas ao lipopolissacarideo (LPS) induzindo inflamação (Pergola, Rossi *et al.*, 2006). Essas evidências sugerem que as ANT podem impedir os mecanismos excitotóxicos relacionados com a síntese de NO, já que o excesso de produção de espécies reativas de nitrogênio (RNS) resulta em estresse nitrosativo, o que contribui para vários processos patológicos subjacentes a doenças neurodegenerativas e inflamatórias (Valko, Leibfritz *et al.*, 2007). A Na^+, K^+-ATPase é um enzima transmembranar sensível a ROS e RNS (Franzon, Lamers *et al.*, 2003) enquanto que a Ca^{2+}-ATPase é uma enzima sensível aos níveis de Ca^{2+} intracelular (Pereira, Ferreira *et al.*, 1996; Huang, Nagaraja *et al.*, 2010). Esta última enzima é a principal moduladora da concentração intracelular deste segundo mensageiro, e aumentos na atividade de Ca^{2+}-ATPase pode ser um mecanismo compensatório para remover um excesso de íon Ca^{2+}, e isto pode ser o resultado da injeção ICV-STZ, uma vez que neste terceiro estudo a atividade da Ca^{2+}-ATPase foi elevada

nesse grupo, diferente do encontrado no modelo de amnésia induzido por SCO. E interessantemente, o tratamento com ANT preveniu estes efeitos deletérios, portanto, a atividade antioxidante das ANT pode estar relacionada com um efeito protetor sobre a disfunção destas enzimas.

Além das propriedades antioxidantes das ANT, não podemos deixar de descartar outros mecanismos neuroprotectores na prevenção do aumento do NOx induzido por ICV-STZ, tais como a afinidade das ANT aos receptores $GABA_A$. Estudos têm investigado que os compostos que potencializam receptores $GABA_A$ (benzodiazepinas) podem impedir o aumento da produção de NO induzido pela administração de NMDA no cerebelo de roedores (Fedele, Ansaldo *et al.*, 2000). Além disso, a ativação dos receptores $GABA_A$ protege os neurônios contra a toxicidade ao peptideo β-amilóide em regiões afetadas no cérebro de animais com DA (Paula-Lima, De Felice *et al.*, 2005).

Uma vez que a regulação do sistema colinérgico, a perda de memória associada com o comportamento ansiogênico tem sido relacionada com a fisiopatologia da DA, os nossos resultados sugerem que as ANT podem exercer ações benéficas, impedindo o aumento da atividade da AChE e perda de memória induzida pelo ICV-STZ. E os resultados obtidos nesse estudo demonstraram, pela primeira vez, que as ANT possuem afinidade para os receptores $GABA_A$, o que pode explicar o efeito ansiolítico *per se* delas em neutralizar o aumento da ansiedade de animais injetados com ICV-STZ. Além disso, as implicações terapêuticas para o uso das ANT pode ser através da sua capacidade de modular a produção de NO e regular a atividade da Na^+, K^+-ATPase e Ca^+-ATPase em situações patológicas.

Outro fato bastante interessante a ser destacado, é que um dos objetivos deste estudo também foi caracterizar modificações morfológicas presentes no hipocampo de animais tratados com ICV-STZ, por esta região cerebral ter um papel central no processamento mnemônico associado aos testes comportamentais de avaliação do desempenho de memória escolhidos (Maia e De Mendonca, 2002; Lynch, 2004). Para verificar as modificações da arquitetura dos neurônios principais do giro dentado e do cornu Ammonis foi utilizando uma coloração de violeta de cresil (Duarte, Carvalho *et al.*, 2009) e também a presença de neurônios lesados utilizando uma coloração com Fluoro Jade-C (Schmued, Stowers *et al.*, 2005) tal como previamente descrito (Canas, Porciuncula *et al.*, 2009). A exposição dos animais a injeção de ICV-STZ por 28 dias foi capaz de alterar a morfologia de células

neuronais confirmadas pela coloração de violeta de cresil (ANEXO I, Figura 1). Nossos resultados revelaram que a administração de ICV-STZ sozinha resultou na diminuição seletiva de viabilidade de neurônios, mais proeminentemente nas regiões da lâmina dorsal do que a região ventral do hipocampo, com a perda de células máxima vista principalmente na região do giro dentado do hipocampo (ANEXO I, Figura 1H). Uma perda de células piramidais na região CA3 também foi observada em todos os animais injetados ICV-STZ (ANEXO I, Figura 1F). Porém, quando verificado lesões e morte de neurônios em animais injetados ICV-STZ pela coloração de Fluoro Jade C, não foi observada qualquer mudança significativa em relação ao grupo controle.

Interessantemente, neste estudo de caracterização morfológica, também resolvemos investigar a densidade de proteinas para a NTPDase 1 e da 5'-nucleotidase por Western Blot, o qual verificamos uma redução na densidade apenas da 5'-NT (ANEXO I, Figura 4B). Uma vez verificado esta mudança de densidade específica para a 5'-NT, resolvemos selecionar esta enzima para confirmar a presença desta no hipocampo, através da sua immunoreatividade utilizando anticorpos específicos conjugados a fluoróforos. Nossos resultados mostraram uma marcante redução na immunoreatividade para a 5'-NT nos animais injetados com ICV-STZ, e mudanças na atividade de ectonucleotidases também foram avaliadas nesse modelo.

O tratamento de maior duração com STZ apresentou uma diminuição significativa para a atividade da NTPDase, utilizando ATP como substrato, e diminuição na atividade da 5'-NT nesses animais (ANEXO I, Figura 4 D, E e F). Além disso, a atividade da 5'-NT mostrou-se alterada em diferentes modelos experimentais estudados por pesquisadores do nosso grupo, em ratos desmielinizados (Spanevello, Mazzanti *et al.*, 2006) e diabéticos (Schmatz, Schetinger *et al.*, 2009) ocorrendo um aumento na sua atividade em sinaptossomas de córtex cerebral. Uma vez que a densidade desta enzima mostrou-se diminuída em animais ICV-STZ tratados, é possível inferir que em situações de demência esta enzima apresenta efeitos mais pronunciados do que a NTPDase 1, indicando que esta enzima pode ter um papel crucial na demência esporádica do tipo Alzheimer, afetando as concentrações de adenosina extracellular, um reconhecido nucleosídeo com propriedades neuromodulatórias e neuroprotetoras. Adicionalmente, outros resultados que corroboram com os anteriores, é que os animais ICV-STZ tratados

mostraram no hipocampo um aumento de até 50,96% maior para os níveis de espécies reativas totais (ROS total) em relação ao controle, também aumento nos níveis de peroxidação de lipídeos (medida do MDA) e de óxido nítrico (NOx), indicando marcante estresse oxidativo e nitrosativo ao qual poderia culminar na diminuição da viabilidade de neurônios, confirmadas pela coloração de violeta de cresil, e diminuição da atividade da NTPDase e 5'-NT, uma vez que elas estão ligadas a membrana celular (Figura 4, B-D)

Curiosamente, a atividade da enzima NTPDase (utilizando ATP como substrato) apresentou uma diminuição significativa no grupo STZ. Apesar da sua densidade não ter sido alterada durando o tratamento com STZ, os resultados da atividade enzimática estão em conformidade com a diminuição dos níveis de ATP verificados por HPLC em animais ICV-STZ tratados. Sabe-se que a piora da função mitocondrial e redução dos níveis de ATP são condições patológicas encontradas em doenças neurodegenerativas, como DA, e isto está intimamente ligada à diminuição dos processos cognitivos (Ferrer, 2009; Hauptmann, Scherping *et al.*, 2009).

No cérebro, o armazenamento da informação está envolvido com um aumento na eficiência de vias estimuladoras sinápticas (Wieraszko e Ehrlich, 1994; Wieraszko, 1996). Desde as primeiras demonstrações de estimulação da libertação ATP (Holton, 1959), existe um interesse crescente no papel da adenosina e do ATP na transmissão sináptica (Cunha e Ribeiro, 2000). ATP e adenosina atuam como neurotransmissores modulando receptores purinérgicos e adenosinérgicos, respectivamente (Zimmermann, 1994). Tem sido relatado que o ATP e adenosina modulam a LTP e LTD em neurônios (De Mendonca, Costenla *et al.*, 2002) e também contribuem para a plasticidade sináptica neuronal (Wieraszko, 1996). Finalmente, estes processos estão relacionados com a aprendizagem e formação da memória (Lovinger, 2010), corroborando com nossos estudos, onde foi possível quantificar o déficit mnemônico de animais ICV-STZ tratados, e esses animais apresentaram perda significativa de memória espacial encontrada na tarefa do labirinto aquático de Morris, e memória sensorial encontrado na tarefa do reconhecimento de objetos como pode ser observado no ANEXO I (Figura 5 A,B e F) desta tese.

8. Conclusões

8.1 Conclusões

- ✓ A administração de ANT foi capaz de antagonizar os déficits de memória induzidos pela injeção intraperitoneal de SCO em ratos.

- ✓ A atividade das enzimas AChE, Na^{+},K^{+}-ATPase e Ca^{2+}-ATPase foram alteradas nos animais injetado com SCO. E o tratamento prévio com ANT reestabeleceu a atividade destas enzimas tanto no córtex cerebral como no hipocampo destes animais. Este efeito preventivo das ANT, juntamente com os parâmetros de memória parece demonstrar que esse composto pode exercer seus mecanismos pela regulação da via colinérgica.

- ✓ Além das alterações na transmissão colinérgica, foi também mostrado que enzimas do sistema purinérgico foram afetadas pela SCO. O aumento na atividade da NTPDase, diminuição na atividade da ADA e 5'-NT no cérebro de animais injetados com SCO pode ser responsável pela redução do ATP e adenosina extracelular na fenda sináptica, ao qual pode comprometer a sinalização purinérgica. E o tratamento com ANT impediu alterações na atividade destas enzimas, podendo assim aumentar a disponibilidade de ATP e adenosina reestabelecendo o funcionamento correto desta via.

- ✓ Quando testamos se as ANT são capazes de prevenir as alterações encontradas num modelo de DAS, induzida por injeção ICV-STZ, nossos resultados mostraram que a injeção de ICV-STZ prejudicou a aquisição de memória dos animais, o tratamento com ANT foi capaz de impedir os déficits de memória encontrados nesse modelo sugerindo um efeito neuroprotetor deste composto.

- ✓ Os animais ICV-STZ tratados apresentaram elevado comportamento ansiogenico e o tratamento com ANT previniu este efeito, indicando que as ANT pode ser considerada um composto com propriedades ansiolíticas, uma vez que experimentos in vitro, mostraram uma afinidade deste composto pelo sítio benzodiazepínico de receptores GABAA.

- ✓ Os animais ICV-STZ tratados apresentaram marcante elevação na atividade da AChE e Ca2+-ATPase, e redução na atividade da Na+,K+-ATPase em ambos tratamentos de curta e longa duração, podemos sugerir que a injeção de ICV-STZ causa uma perturbação nas concentrações eletrolíticas de Na+ e de Ca2+ e isto poderia levar a excitotoxicidade neuronal e aumento na atividade da AChE no modelo experimental para a DAS, e estes efeitos podem ser prevenidos, pelo menos, a curto prazo com a administração de ANT, como observado neste estudo.

- ✓ Os animais ICV-STZ tratados por 28 dias apresentaram modificações morfológicas em células neuronais, indicando diminuição seletiva de viabilidade de neuronios, mais especificamente nas regiões CA1, CA3 e giro dentado do hipocampo em comparação com animais normais. Porém, a injeção ICV-STZ não foi responsável por lesões que levariam a morte dos neuronios.

- ✓ Tanto a densidade como a immunoreatividade para a 5'-NT foi marcadamente reduzida em animais ICV-STZ tratados em comparação com o grupo de ratos normais. Além disso, o tratamento com STZ mostrou uma diminuição significativa para a atividade da NTPDase e 5'-NT. Uma vez que a densidade da 5'-NT mostrou-se diminuída em animais ICV-STZ tratados, é possível sugerir que em situações de demência esta enzima apresenta efeitos mais pronunciados do que a NTPDase 1, indicando que a 5'-NT pode ter um papel importante na DAS.

- ✓ No hipocampo de animais ICV-STZ tratados foi encontrado uma elevação considerável nos níveis de ROS total, MDA e NOx, indicando marcante estresse oxidativo, que poderia levar a diminuição na viabilidade de neurônios e na atividade das enzimas NTPDase e 5’-NT, uma vez que elas estão ligadas a membrana celular.

Referências

REFERÊNCIAS

Abbracchio, M. P. e G. Burnstock. Purinergic signalling: pathophysiological roles. Jpn J Pharmacol, v.78, n.2, Oct, p.113-45. 1998.

Abe, L. T. Phenolic compounds and antioxidant activity of *Vitislabrusca* and *Vitisvinifera cultivars*. Food Science and Technology, v.27, n.2, p.394-400. 2007.

Adlard, P. A., S. A. James*, et al.* beta-Amyloid as a molecular therapeutic target in Alzheimer's disease. Drugs Today (Barc), v.45, n.4, Apr, p.293-304. 2009.

Agrawal, R., E. Tyagi*, et al.* A study of brain insulin receptors, AChE activity and oxidative stress in rat model of ICV STZ induced dementia. Neuropharmacology, v.56, n.4, Mar, p.779-87. 2009.

Agteresch, H. J., P. C. Dagnelie*, et al.* Adenosine triphosphate: established and potential clinical applications. Drugs, v.58, n.2, Aug, p.211-32. 1999.

Ali, E. H. e N. M. Arafa. Comparative protective action of curcumin, memantine and diclofenac against scopolamine-induced memory dysfunction. Fitoterapia, v.82, n.4, Jun, p.601-8. 2011.

Anagnostaras, S. G., S. A. Josselyn*, et al.* Computer-assisted behavioral assessment of Pavlovian fear conditioning in mice. Learn Mem, v.7, n.1, Jan, p.58-72. 2000.

Andersen, Ø. M. e K. R. Markham. FLAVONOIDS: Chemistry, Biochemistry and Applications. CRC Press, Taylor & Francis Group, Boca Raton, FL p.471-551. 2006.

Andres-Lacueva, C., B. Shukitt-Hale*, et al.* Anthocyanins in aged blueberry-fed rats are found centrally and may enhance memory. Nutr Neurosci, v.8, n.2, Apr, p.111-20. 2005.

Appleyard, M. E. Non-cholinergic functions of acetylcholinesterase. Biochem Soc Trans, v.22, n.3, Aug, p.749-55. 1994.

Archibald, J. T. e T. D. White. Rapid reversal of internal Na+ and K+ contents of synaptosomes by ouabain. Nature, v.252, n.5484, Dec 13, p.595-6. 1974.

Awasthi, H., S. Tota*, et al.* Protective effect of curcumin against intracerebral streptozotocin induced impairment in memory and cerebral blood flow. Life Sci, v.86, n.3-4, Jan 16, p.87-94. 2010.

Bao, L., X. S. Yao*, et al.* Protective effects of bilberry (Vaccinium myrtillus L.) extract on KBrO3-induced kidney damage in mice. J Agric Food Chem, v.56, n.2, Jan 23, p.420-5. 2008.

Bao, L., X. S. Yao*, et al.* Protective effects of bilberry (Vaccinium myrtillus L.) extract on restraint stress-induced liver damage in mice. J Agric Food Chem, v.56, n.17, Sep 10, p.7803-7. 2008.

Barros, D., O. B. Amaral*, et al.* Behavioral and genoprotective effects of Vaccinium berries intake in mice. Pharmacol Biochem Behav, v.84, n.2, Jun, p.229-34. 2006.

Barzilai, N., G. Atzmon*, et al.* A genotype of exceptional longevity is associated with preservation of cognitive function. Neurology, v.67, n.12, Dec 26, p.2170-5. 2006.

Belzung, C. e G. Griebel. Measuring normal and pathological anxiety-like behaviour in mice: a review. Behav Brain Res, v.125, n.1-2, Nov 1, p.141-9. 2001.

Benarroch, E. E. Na+, K+-ATPase: functions in the nervous system and involvement in neurologic disease. Neurology, v.76, n.3, Jan 18, p.287-93. 2011.

Berchtold, N. C. e C. W. Cotman. Evolution in the conceptualization of dementia and Alzheimer's disease: Greco-Roman period to the 1960s. Neurobiol Aging, v.19, n.3, May-Jun, p.173-89. 1998.

Berlese, D. B., P. D. Sauzem*, et al.* Time-dependent modulation of inhibitory avoidance memory by spermidine in rats. Neurobiol Learn Mem, v.83, n.1, Jan, p.48-53. 2005.

Bernardi, L., S. Geracitano*, et al.* AbetaPP A713T mutation in late onset Alzheimer's disease with cerebrovascular lesions. J Alzheimers Dis, v.17, n.2, p.383-9. 2009.

Berrocal, M., D. Marcos*, et al.* Altered Ca2+ dependence of synaptosomal plasma membrane Ca2+-ATPase in human brain affected by Alzheimer's disease. FASEB J, v.23, n.6, Jun, p.1826-34. 2009.

Bianchi, V. e J. Spychala. Mammalian 5'-nucleotidases. J Biol Chem, v.278, n.47, Nov 21, p.46195-8. 2003.

Bishayee, A., E. Haznagy-Radnai*, et al.* Anthocyanin-rich black currant extract suppresses the growth of human hepatocellular carcinoma cells. Nat Prod Commun, v.5, n.10, Oct, p.1613-8. 2010.

Blin, J., M. F. Piercey*, et al.* Metabolic effects of scopolamine and physostigmine in human brain measured by positron emission tomography. J Neurol Sci, v.123, n.1-2, May, p.44-51. 1994.

Blitzer, R. D., O. Gil*, et al.* Cholinergic stimulation enhances long-term potentiation in the CA1 region of rat hippocampus. Neurosci Lett, v.119, n.2, Nov 13, p.207-10. 1990.

Blokland, A. Acetylcholine: a neurotransmitter for learning and memory? Brain Res Brain Res Rev, v.21, n.3, Nov, p.285-300. 1995.

Blokland, A. e J. Jolles. Spatial learning deficit and reduced hippocampal ChAT activity in rats after an ICV injection of streptozotocin. Pharmacol Biochem Behav, v.44, n.2, Feb, p.491-4. 1993.

Bodin, P. e G. Burnstock. Purinergic signalling: ATP release. Neurochem Res, v.26, n.8-9, Sep, p.959-69. 2001.

Boldyrev, A., E. Bulygina*, et al.* Glutamate receptors communicate with Na+/K+-ATPase in rat cerebellum granule cells: demonstration of differences in the action of several metabotropic and ionotropic glutamate agonists on intracellular reactive oxygen species and the sodium pump. J Mol Neurosci, v.21, n.3, p.213-22. 2003.

Boldyrev, A., E. Bulygina*, et al.* Functional relationship between Na/K-ATPase and NMDA-receptors in rat cerebellum granule cells. Biochemistry (Mosc), v.69, n.4, Apr, p.429-34. 2004.

Boldyrev, A. A. e E. R. Bulygina. Na/K-ATPase and oxidative stress. Ann N Y Acad Sci, v.834, Nov 3, p.666-8. 1997.

Boldyrev, A. A., E. R. Bulygina*, et al.* Effect of nitroso compounds on Na/K-ATPase. Biochim Biophys Acta, v.1321, n.3, Oct 20, p.243-51. 1997.

Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem, v.72, May 7, p.248-54. 1976.

Bravo, L. Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. Nutr Rev, v.56, n.11, Nov, p.317-33. 1998.

Brines, M. L. e R. J. Robbins. Inhibition of alpha 2/alpha 3 sodium pump isoforms potentiates glutamate neurotoxicity. Brain Res, v.591, n.1, Sep 18, p.94-102. 1992.

Brunelli, M., M. Garcia-Gil*, et al.* Neurobiological principles of learning and memory. Arch Ital Biol, v.135, n.1, Jan, p.15-36. 1997.

Burnstock, G. Historical review: ATP as a neurotransmitter. Trends Pharmacol Sci, v.27, n.3, Mar, p.166-76. 2006.

Burnstock, G. e C. Kennedy. Is there a basis for distinguishing two types of P2-purinoceptor? Gen Pharmacol, v.16, n.5, p.433-40. 1985.

Bymaster, F. P., P. A. Carter*, et al.* Xanomeline compared to other muscarinic agents on stimulation of phosphoinositide hydrolysis in vivo and other cholinomimetic effects. Brain Res, v.795, n.1-2, Jun 8, p.179-90. 1998.

Bymaster, F. P., H. E. Shannon*, et al.* Unexpected antipsychotic-like activity with the muscarinic receptor ligand (5R,6R)6-(3-propylthio-1,2,5-thiadiazol-4-yl)-1-azabicyclo[3.2.1]octane. Eur J Pharmacol, v.356, n.2-3, Sep 4, p.109-19. 1998.

Canadian study of health and aging: study methods and prevalence of dementia. CMAJ, v.150, n.6, Mar 15, p.899-913. 1994.

Canas, P. M., L. O. Porciuncula*, et al.* Adenosine A2A receptor blockade prevents synaptotoxicity and memory dysfunction caused by beta-amyloid peptides via p38

mitogen-activated protein kinase pathway. J Neurosci, v.29, n.47, Nov 25, p.14741-51. 2009.

Candore, G., C. R. Balistreri*, et al.* Immunogenetics, gender, and longevity. Ann N Y Acad Sci, v.1089, Nov, p.516-37. 2006.

Carta, M., R. Stancampiano*, et al.* Vitamin A deficiency induces motor impairments and striatal cholinergic dysfunction in rats. Neuroscience, v.139, n.4, p.1163-72. 2006.

Carvalho, F. B., C. F. Mello*, et al.* Spermidine decreases Na(+),K(+)-ATPase activity through NMDA receptor and protein kinase G activation in the hippocampus of rats. Eur J Pharmacol, v.684, n.1-3, Jun 5, p.79-86. 2012.

Castañeda-Ovando, A., M. Pacheco-Hernández*, et al.* Chemical studies of anthocyanins: A review. Food Chemistry v.113 p.859-871. 2009.

Casteels, R., F. Wuytack*, et al.* Ca(2+)-transport ATPases and Ca(2+)-compartments in smooth muscle cells. Z Kardiol, v.80 Suppl 7, p.65-8. 1991.

Chacon, M. A., A. E. Reyes*, et al.* Acetylcholinesterase induces neuronal cell loss, astrocyte hypertrophy and behavioral deficits in mammalian hippocampus. J Neurochem, v.87, n.1, Oct, p.195-204. 2003.

Chauhan, N. e G. Siegel. Differential expression of Na,K-ATPase alpha-isoform mRNAs in aging rat cerebellum. J Neurosci Res, v.47, n.3, Feb 1, p.287-99. 1997a.

_____. Na,K-ATPase: increases in alpha1-messenger RNA and decreases in alpha3-messenger RNA levels in aging rat cerebral cortex. Neuroscience, v.78, n.1, May, p.7-11. 1997b.

Chauhan, N. B., J. M. Lee*, et al.* Na,K-ATPase mRNA levels and plaque load in Alzheimer's disease. J Mol Neurosci, v.9, n.3, Dec, p.151-66. 1997.

Chin, J. H., F. W. Tse*, et al.* Beta-amyloid enhances intracellular calcium rises mediated by repeated activation of intracellular calcium stores and nicotinic receptors in acutely dissociated rat basal forebrain neurons. Brain Cell Biol, v.35, n.2-3, Jun, p.173-86. 2006.

Choi, D. Y., Y. J. Lee*, et al.* Attenuation of scopolamine-induced cognitive dysfunction by obovatol. Arch Pharm Res, v.35, n.7, Jul, p.1279-86. 2012.

Choi, E. H., H. E. Ok*, et al.* Protective effect of anthocyanin-rich extract from bilberry (Vaccinium myrtillus L.) against myelotoxicity induced by 5-fluorouracil. Biofactors, v.29, n.1, p.55-65. 2007.

Christensen, H., N. Maltby*, et al.* Cholinergic 'blockade' as a model of the cognitive deficits in Alzheimer's disease. Brain, v.115 (Pt 6), Dec, p.1681-99. 1992.

Clarke, R. J. e X. Fan. Pumping ions. Clin Exp Pharmacol Physiol, v.38, n.11, Nov, p.726-33. 2011.

Corrada, M. M., R. Brookmeyer*, et al.* Prevalence of dementia after age 90: results from the 90+ study. Neurology, v.71, n.5, Jul 29, p.337-43. 2008.

Cunha, R. A. Adenosine as a neuromodulator and as a homeostatic regulator in the nervous system: different roles, different sources and different receptors. Neurochem Int, v.38, n.2, Feb, p.107-25. 2001.

Cunha, R. A. e J. A. Ribeiro. ATP as a presynaptic modulator. Life Sci, v.68, n.2, Dec 1, p.119-37. 2000.

Curtright, R., R. Emry*, et al.* Facilitating student understanding of buffering by an integration of mathematics and chemical concepts. Biochem Mol Biol Educ, v.32, n.2, Mar, p.71-7. 2004.

Da Silva, R. S., S. K. Richetti*, et al.* Maternal caffeine intake affects acetylcholinesterase in hippocampus of neonate rats. Int J Dev Neurosci, v.26, n.3-4, May-Jun, p.339-43. 2008.

Darvesh, A. S., R. T. Carroll*, et al.* Oxidative stress and Alzheimer's disease: dietary polyphenols as potential therapeutic agents. Expert Rev Neurother, v.10, n.5, May, p.729-45. 2010.

De Bruin, N. e B. Pouzet. Beneficial effects of galantamine on performance in the object recognition task in Swiss mice: deficits induced by scopolamine and by prolonging the retention interval. Pharmacol Biochem Behav, v.85, n.1, Sep, p.253-60. 2006.

De La Monte, S. M., A. Neusner*, et al.* Epidemilogical trends strongly suggest exposures as etiologic agents in the pathogenesis of sporadic Alzheimer's disease, diabetes mellitus, and non-alcoholic steatohepatitis. J Alzheimers Dis, v.17, n.3, p.519-29. 2009.

De La Monte, S. M., M. Tong*, et al.* Therapeutic rescue of neurodegeneration in experimental type 3 diabetes: relevance to Alzheimer's disease. J Alzheimers Dis, v.10, n.1, Sep, p.89-109. 2006.

De La Torre, J. C. Pathophysiology of neuronal energy crisis in Alzheimer's disease. Neurodegener Dis, v.5, n.3-4, p.126-32. 2008.

De Mendonca, A., A. R. Costenla*, et al.* Persistence of the neuromodulatory effects of adenosine on synaptic transmission after long-term potentiation and long-term depression. Brain Res, v.932, n.1-2, Apr 5, p.56-60. 2002.

Del Rio, D., G. Borges*, et al.* Berry flavonoids and phenolics: bioavailability and evidence of protective effects. Br J Nutr, v.104 Suppl 3, Oct, p.S67-90. 2010.

Dey, P. M. e J. B. Harborne. Plant phenolics methods in plant biochemistry. London: Academic Press Limited., v.2, n.326-341. 1993.

Dipolo, R. e L. Beauge. Regulation of Na-Ca exchange. An overview. Ann N Y Acad Sci, v.639, p.100-11. 1991.

Dominguez, L. J. e M. Barbagallo. The cardiometabolic syndrome and sarcopenic obesity in older persons. J Cardiometab Syndr, v.2, n.3, Summer, p.183-9. 2007.

Dos Reis, E. A., L. S. De Oliveira*, et al.* Arginine administration inhibits hippocampal Na(+),K(+)-ATPase activity and impairs retention of an inhibitory avoidance task in rats. Brain Res, v.951, n.2, Oct 4, p.151-7. 2002.

Droge, W. e R. Kinscherf. Aberrant insulin receptor signaling and amino acid homeostasis as a major cause of oxidative stress in aging. Antioxid Redox Signal, v.10, n.4, Apr, p.661-78. 2008.

Duarte, J. M., R. A. Carvalho*, et al.* Caffeine consumption attenuates neurochemical modifications in the hippocampus of streptozotocin-induced diabetic rats. J Neurochem, v.111, n.2, Oct, p.368-79. 2009.

Duffy, K. B., E. L. Spangler*, et al.* A blueberry-enriched diet provides cellular protection against oxidative stress and reduces a kainate-induced learning impairment in rats. Neurobiol Aging, v.29, n.11, Nov, p.1680-9. 2008.

East, S. J. e J. Garthwaite. NMDA receptor activation in rat hippocampus induces cyclic GMP formation through the L-arginine-nitric oxide pathway. Neurosci Lett, v.123, n.1, Feb 11, p.17-9. 1991.

Edirisinghe, I., K. Banaszewski*, et al.* Effect of black currant anthocyanins on the activation of endothelial nitric oxide synthase (eNOS) in vitro in human endothelial cells. J Agric Food Chem, v.59, n.16, Aug 24, p.8616-24. 2011.

El-Sherbiny, D. A., A. E. Khalifa*, et al.* Hypericum perforatum extract demonstrates antioxidant properties against elevated rat brain oxidative status induced by amnestic dose of scopolamine. Pharmacol Biochem Behav, v.76, n.3-4, Dec, p.525-33. 2003.

Ellman, G. L., K. D. Courtney*, et al.* A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol, v.7, Jul, p.88-95. 1961.

Ennaceur, A. e K. Meliani. Effects of physostigmine and scopolamine on rats' performances in object-recognition and radial-maze tests. Psychopharmacology (Berl), v.109, n.3, p.321-30. 1992.

Erecinska, M. e I. A. Silver. Tissue oxygen tension and brain sensitivity to hypoxia. Respir Physiol, v.128, n.3, Nov 15, p.263-76. 2001.

Fakira, A. K., L. D. Gaspers*, et al.* Purkinje cell dysfunction and delayed death in plasma membrane calcium ATPase 2-heterozygous mice. Mol Cell Neurosci, v.51, n.1-2, Aug, p.22-31. 2012.

Fedele, E., M. A. Ansaldo*, et al.* Benzodiazepine-sensitive GABA(A) receptors limit the activity of the NMDA/NO/cyclic GMP pathway: a microdialysis study in the cerebellum of freely moving rats. J Neurochem, v.75, n.2, Aug, p.782-7. 2000.

Felgines, C., O. Texier*, et al.* Blackberry anthocyanins are slightly bioavailable in rats. J Nutr, v.132, n.6, Jun, p.1249-53. 2002.

Ferreira, G., R. Gervais*, et al.* Postacquisition scopolamine treatments reveal the time course for the formation of lamb odor recognition memory in parturient ewes. Behav Neurosci, v.113, n.1, Feb, p.136-42. 1999.

Ferrer, I. Altered mitochondria, energy metabolism, voltage-dependent anion channel, and lipid rafts converge to exhaust neurons in Alzheimer's disease. J Bioenerg Biomembr, v.41, n.5, Oct, p.425-31. 2009.

Fields, R. D. e G. Burnstock. Purinergic signalling in neuron-glia interactions. Nat Rev Neurosci, v.7, n.6, Jun, p.423-36. 2006.

Fiske, C. H. e Y. Subbarow. The Nature of the "Inorganic Phosphate" in Voluntary Muscle. Science, v.65, n.1686, Apr 22, p.401-3. 1927.

Frank, J., A. Kamal-Eldin*, et al.* Effects of dietary anthocyanins on tocopherols and lipids in rats. J Agric Food Chem, v.50, n.25, Dec 4, p.7226-30. 2002.

Franzon, R., M. L. Lamers*, et al.* Evidence that oxidative stress is involved in the inhibitory effect of proline on Na(+),K(+)-ATPase activity in synaptic plasma membrane of rat hippocampus. Int J Dev Neurosci, v.21, n.6, Oct, p.303-7. 2003.

Frussa-Filho, R., H. Barbosa-Junior*, et al.* Naltrexone potentiates the anxiolytic effects of chlordiazepoxide in rats exposed to novel environments. Psychopharmacology (Berl), v.147, n.2, Nov, p.168-73. 1999.

Fuchs, J. L. 5'-Nucleotidase activity increases in aging rat brain. Neurobiol Aging, v.12, n.5, Sep-Oct, p.523-30. 1991.

Fujisawa, H., K. Kajikawa*, et al.* Movement of radioactive calcium in brain slices and influences on it of protoveratrine, ouabain, potassium chloride and cocaine. Jpn J Pharmacol, v.15, n.4, Dec, p.327-34. 1965.

Gao, S., H. C. Hendrie*, et al.* The relationships between age, sex, and the incidence of dementia and Alzheimer disease: a meta-analysis. Arch Gen Psychiatry, v.55, p.809-815. 1998.

Garcia-Beneytez, E., F. Cabello*, et al.* Analysis of grape and wine anthocyanins by HPLC-MS. J Agric Food Chem, v.51, n.19, Sep 10, p.5622-9. 2003.

Geering, K. Subunit assembly and functional maturation of Na,K-ATPase. J Membr Biol, v.115, n.2, May, p.109-21. 1990.

_____. Na,K-ATPase. Curr Opin Nephrol Hypertens, v.6, n.5, Sep, p.434-9. 1997.

Gether, U., P. H. Andersen*, et al.* Neurotransmitter transporters: molecular function of important drug targets. Trends Pharmacol Sci, v.27, n.7, Jul, p.375-83. 2006.

Giacobini, E. New trends in cholinergic therapy for Alzheimer disease: nicotinic agonists or cholinesterase inhibitors? Prog Brain Res, v.109, p.311-23. 1996.

Gibbs, M. E. a. N., Kim T. Psychobiology of memory: Towards a model of memory formation. Biobehavioral Rewiews, v.1, n.2, p.113-136. 2003.

Giusti, M. M., L. E. Rodriguez-Saona*, et al.* Electrospray and tandem mass spectroscopy as tools for anthocyanin characterization. J Agric Food Chem, v.47, n.11, Nov, p.4657-64. 1999.

Giusti, M. M. e R. E. Wrolstad. Anthocyanins: Characterization and measurement of anthocyanins by UV–Visible spectroscopy. Current protocols in food analytical chemistry. , v.1, n.2, p.1-13. 2001.

Glushchenko, T. S. e N. L. Izvarina. Na+,K(+)-ATPase activity in neurons and glial cells of the olfactory cortex of the rat brain during the development of long-term potentiation. Neurosci Behav Physiol, v.27, n.1, Jan-Feb, p.49-52. 1997.

Gold, P. E. Acetylcholine modulation of neural systems involved in learning and memory. Neurobiol Learn Mem, v.80, n.3, Nov, p.194-210. 2003.

Gonzalez-Paramas, A. M., S. Esteban-Ruano*, et al.* Flavanol content and antioxidant activity in winery byproducts. J Agric Food Chem, v.52, n.2, Jan 28, p.234-8. 2004.

Gouaux, E. Review. The molecular logic of sodium-coupled neurotransmitter transporters. Philos Trans R Soc Lond B Biol Sci, v.364, n.1514, Jan 27, p.149-54. 2009.

Greene, J. G. e J. T. Greenamyre. Manipulation of membrane potential modulates malonate-induced striatal excitotoxicity in vivo. J Neurochem, v.66, n.2, Feb, p.637-43. 1996.

Grisar, T., D. Guillaume*, et al.* Contribution of Na+,K(+)-ATPase to focal epilepsy: a brief review. Epilepsy Res, v.12, n.2, Jul, p.141-9. 1992.

Grisaru, D., M. Sternfeld*, et al.* Structural roles of acetylcholinesterase variants in biology and pathology. Eur J Biochem, v.264, n.3, Sep, p.672-86. 1999.

Grobben, B., K. Anciaux*, et al.* An ecto-nucleotide pyrophosphatase is one of the main enzymes involved in the extracellular metabolism of ATP in rat C6 glioma. J Neurochem, v.72, n.2, Feb, p.826-34. 1999.

Gron, G., I. Brandenburg*, et al.* Inhibition of hippocampal function in mild cognitive impairment: targeting the cholinergic hypothesis. Neurobiol Aging, v.27, n.1, Jan, p.78-87. 2006.

Grunblatt, E., M. Salkovic-Petrisic*, et al.* Brain insulin system dysfunction in streptozotocin intracerebroventricularly treated rats generates hyperphosphorylated tau protein. J Neurochem, v.101, n.3, May, p.757-70. 2007.

Gutierres, J. M., F. B. Carvalho*, et al.* Protective effect of α-Tocopherol on memory deficits and Na+,K+-ATPase and acetylcholinesterase activities in rats with diet-induced hypercholesterolemia. Biomedicine & Aging Pathology, v.2, n.3, p.73-80. 2012.

Gutierres, J. M., F. B. Carvalho*, et al.* Protective effects of anthocyanins on the ectonucleotidase activity in the impairment of memory induced by scopolamine in adult rats. Life Sci, v.91, n.23-24, Dec 10, p.1221-8. 2012.

Gutierres, J. M., R. R. Kaizer*, et al.* alpha-Tocopherol regulates ectonucleotidase activities in synaptosomes from rats fed a high-fat diet. Cell Biochem Funct, v.30, n.4, Jun, p.286-92. 2012a.

_____. alpha-Tocopherol regulates ectonucleotidase activities in synaptosomes from rats fed a high-fat diet. Cell Biochem Funct, Jan 6. 2012b.

Harborne, J. B. e C. A. Williams. Advances in flavonoid research since 1992. Phytochemistry, v.55, n.6, p.481-504. 2000.

_____. Anthocyanins and other flavonoids. Nat Prod Rep, v.18, n.3, Jun, p.310-33. 2001.

Hattori, N., K. Kitagawa*, et al.* CI-ATPase and Na+/K(+)-ATPase activities in Alzheimer's disease brains. Neurosci Lett, v.254, n.3, Oct 2, p.141-4. 1998.

Hauptmann, S., I. Scherping*, et al.* Mitochondrial dysfunction: an early event in Alzheimer pathology accumulates with age in AD transgenic mice. Neurobiol Aging, v.30, n.10, Oct, p.1574-86. 2009.

Heinonen, M. Anthocyanins as dietary antioxidants. Third international conference on natural antioxidants and anticarcinogens in food, health and disease (NADH), n.25, p.6-9. 2001.

Holton, P. The liberation of adenosine triphosphate on antidromic stimulation of sensory nerves. J Physiol, v.145, n.3, Mar 12, p.494-504. 1959.

Honer, W. G., I. Prohovnik*, et al.* Scopolamine reduces frontal cortex perfusion. J Cereb Blood Flow Metab, v.8, n.5, Oct, p.635-41. 1988.

Hoyer, S. Causes and consequences of disturbances of cerebral glucose metabolism in sporadic Alzheimer disease: therapeutic implications. Adv Exp Med Biol, v.541, p.135-52. 2004a.

_____. Glucose metabolism and insulin receptor signal transduction in Alzheimer disease. Eur J Pharmacol, v.490, n.1-3, Apr 19, p.115-25. 2004b.

Hoyer, S., H. Lannert*, et al.* Damaged neuronal energy metabolism and behavior are improved by Ginkgo biloba extract (EGb 761). J Neural Transm, v.106, n.11-12, p.1171-88. 1999.

Huang, H., R. Y. Nagaraja*, et al.* Contribution of plasma membrane Ca ATPase to cerebellar synapse function. World J Biol Chem, v.1, n.5, May 26, p.95-102. 2010.

Huang, H. C. e Z. F. Jiang. Accumulated amyloid-beta peptide and hyperphosphorylated tau protein: relationship and links in Alzheimer's disease. J Alzheimers Dis, v.16, n.1, p.15-27. 2009.

Hunsaker, M. R., J. L. Rogers*, et al.* Behavioral characterization of a transection of dorsal CA3 subcortical efferents: comparison with scopolamine and physostigmine infusions into dorsal CA3. Neurobiol Learn Mem, v.88, n.1, Jul, p.127-36. 2007.

Hut, R. A. e E. A. Van Der Zee. The cholinergic system, circadian rhythmicity, and time memory. Behav Brain Res, v.221, n.2, Aug 10, p.466-80. 2011.

Ibach, B. e E. Haen. Acetylcholinesterase inhibition in Alzheimer's Disease. Curr Pharm Des, v.10, n.3, p.231-51. 2004.

Izquierdo, I. Mechanism of action of scopolamine as an amnestic. Trends Pharmacol Sci, v.10, n.5, May, p.175-7. 1989.

Jamme, I., E. Petit*, et al.* Modulation of mouse cerebral Na+,K(+)-ATPase activity by oxygen free radicals. Neuroreport, v.7, n.1, Dec 29, p.333-7. 1995.

Jeong, E. J., K. Y. Lee*, et al.* Cognitive-enhancing and antioxidant activities of iridoid glycosides from Scrophularia buergeriana in scopolamine-treated mice. Eur J Pharmacol, v.588, n.1, Jun 24, p.78-84. 2008.

Jimenez, T., G. Sanchez*, et al.* Activity of the Na,K-ATPase alpha4 isoform is important for membrane potential, intracellular Ca2+, and pH to maintain motility in rat spermatozoa. Reproduction, v.139, n.5, May, p.835-45. 2010.

Jones, C. K., N. Byun*, et al.* Muscarinic and nicotinic acetylcholine receptor agonists and allosteric modulators for the treatment of schizophrenia. Neuropsychopharmacology, v.37, n.1, Jan, p.16-42. 2012.

Jorgensen, P. L., K. O. Hakansson*, et al.* Structure and mechanism of Na,K-ATPase: functional sites and their interactions. Annu Rev Physiol, v.65, p.817-49. 2003.

Joseph, J. A., B. Shukitt-Hale*, et al.* Reversing the deleterious effects of aging on neuronal communication and behavior: beneficial properties of fruit polyphenolic compounds. Am J Clin Nutr, v.81, n.1 Suppl, Jan, p.313S-316S. 2005.

Juranic, Z. e Z. Zizak. Biological activities of berries: from antioxidant capacity to anti-cancer effects. Biofactors, v.23, n.4, p.207-11. 2005.

Kahkonen, M. P. e M. Heinonen. Antioxidant activity of anthocyanins and their aglycons. J Agric Food Chem, v.51, n.3, Jan 29, p.628-33. 2003.

Kahkonen, M. P., A. I. Hopia*, et al.* Berry phenolics and their antioxidant activity. J Agric Food Chem, v.49, n.8, Aug, p.4076-82. 2001.

Kalt, W., J. B. Blumberg*, et al.* Identification of anthocyanins in the liver, eye, and brain of blueberry-fed pigs. J Agric Food Chem, v.56, n.3, Feb 13, p.705-12. 2008.

Kaplan, J. H. Biochemistry of Na,K-ATPase. Annu Rev Biochem, v.71, p.511-35. 2002.

Kasa, P., Z. Rakonczay*, et al.* The cholinergic system in Alzheimer's disease. Prog Neurobiol, v.52, n.6, Aug, p.511-35. 1997.

Ke, Z., Y. Liu*, et al.* Cyanidin-3-glucoside ameliorates ethanol neurotoxicity in the developing brain. J Neurosci Res, v.89, n.10, Oct, p.1676-84. 2011.

Kim, H. G., M. S. Ju*, et al.* Mulberry fruit protects dopaminergic neurons in toxin-induced Parkinson's disease models. Br J Nutr, v.104, n.1, Jul, p.8-16. 2010.

Kim, H. P., K. H. Son*, et al.* Anti-inflammatory plant flavonoids and cellular action mechanisms. J Pharmacol Sci, v.96, n.3, Nov, p.229-45. 2004.

Kirvela, O. A., J. H. Kanto*, et al.* Anticholinergic drugs: effects on oxygen consumption and energy expenditure. Anesth Analg, v.78, n.5, May, p.995-9. 1994.

Klinkenberg, I. e A. Blokland. The validity of scopolamine as a pharmacological model for cognitive impairment: a review of animal behavioral studies. Neurosci Biobehav Rev, v.34, n.8, Jul, p.1307-50. 2010.

Klinkenberg, I., A. Sambeth*, et al.* Acetylcholine and attention. Behav Brain Res, v.221, n.2, Aug 10, p.430-42. 2011.

Kodavanti, P. R. Measurement of calcium buffering by intracellular organelles in brain. Methods Mol Med, v.22, p.171-6. 1999.

Kong, J. M., L. S. Chia*, et al.* Analysis and biological activities of anthocyanins. Phytochemistry, v.64, n.5, Nov, p.923-33. 2003.

Kono, R., N. Tashiro*, et al.* Inhibitory effects of acetylcholine on aggressive-defense reaction induced by electrical stimulation of the hypothalamus in cats. Brain Res Bull, v.16, n.4, Apr, p.491-5. 1986.

Kopelman, M. D. e T. H. Corn. Cholinergic 'blockade' as a model for cholinergic depletion. A comparison of the memory deficits with those of Alzheimer-type dementia and the alcoholic Korsakoff syndrome. Brain, v.111 (Pt 5), Oct, p.1079-110. 1988.

Krikorian, R., E. L. Boespflug*, et al.* Concord Grape Juice Supplementation and Neurocognitive Function in Human Aging. J Agric Food Chem, Apr 9. 2012.

Krikorian, R., J. C. Eliassen*, et al.* Improved cognitive-cerebral function in older adults with chromium supplementation. Nutr Neurosci, v.13, n.3, Jun, p.116-22. 2010.

Krikorian, R., M. D. Shidler*, et al.* Blueberry supplementation improves memory in older adults. J Agric Food Chem, v.58, n.7, Apr 14, p.3996-4000. 2010.

Krueger, L. E. e T. A. Salthouse. Influence of cognitive abilities and age on word recall performance across trials and list segments. Am J Psychol, v.124, n.3, Fall, p.291-300. 2011.

Kumar, A. R. e P. A. Kurup. Inhibition of membrane Na+-K+ ATPase activity: a common pathway in central nervous system disorders. J Assoc Physicians India, v.50, Mar, p.400-6. 2002.

Lambert, G. Caravaggio. Editora Taschen, p.220. 2012.

Lane, R. M., S. G. Potkin*, et al.* Targeting acetylcholinesterase and butyrylcholinesterase in dementia. Int J Neuropsychopharmacol, v.9, n.1, Feb, p.101-24. 2006.

Lannert, H. e S. Hoyer. Intracerebroventricular administration of streptozotocin causes long-term diminutions in learning and memory abilities and in cerebral energy metabolism in adult rats. Behav Neurosci, v.112, n.5, Oct, p.1199-208. 1998.

Lees, G. J., A. Lehmann*, et al.* The neurotoxicity of ouabain, a sodium-potassium ATPase inhibitor, in the rat hippocampus. Neurosci Lett, v.120, n.2, Dec 11, p.159-62. 1990.

Lester-Coll, N., E. J. Rivera*, et al.* Intracerebral streptozotocin model of type 3 diabetes: relevance to sporadic Alzheimer's disease. J Alzheimers Dis, v.9, n.1, Mar, p.13-33. 2006.

Li, S. e P. K. Stys. Na(+)-K(+)-ATPase inhibition and depolarization induce glutamate release via reverse Na(+)-dependent transport in spinal cord white matter. Neuroscience, v.107, n.4, p.675-83. 2001.

Liguri, G., N. Taddei*, et al.* Changes in Na+,K(+)-ATPase, Ca2(+)-ATPase and some soluble enzymes related to energy metabolism in brains of patients with Alzheimer's disease. Neurosci Lett, v.112, n.2-3, May 4, p.338-42. 1990.

Lingrel, J. B. e T. Kuntzweiler. Na+,K(+)-ATPase. J Biol Chem, v.269, n.31, Aug 5, p.19659-62. 1994.

Lingrel, J. B., J. Van Huysse*, et al.* Structure-function studies of the Na,K-ATPase. Kidney Int Suppl, v.44, Jan, p.S32-9. 1994.

Lingrel, J. B., M. T. Williams*, et al.* Na,K-ATPase and the role of alpha isoforms in behavior. J Bioenerg Biomembr, v.39, n.5-6, Dec, p.385-9. 2007.

Lipton, S. A., Y. B. Choi*, et al.* A redox-based mechanism for the neuroprotective and neurodestructive effects of nitric oxide and related nitroso-compounds. Nature, v.364, n.6438, Aug 12, p.626-32. 1993.

Lipton, S. A., D. J. Singel*, et al.* Nitric oxide in the central nervous system. Prog Brain Res, v.103, p.359-64. 1994.

Liu, Y., F. Liu*, et al.* Deficient brain insulin signalling pathway in Alzheimer's disease and diabetes. J Pathol, v.225, n.1, Sep, p.54-62. 2011.

Liu, Y., F. Liu*, et al.* Decreased glucose transporters correlate to abnormal hyperphosphorylation of tau in Alzheimer disease. FEBS Lett, v.582, n.2, Jan 23, p.359-64. 2008.

Lovinger, D. M. Neurotransmitter roles in synaptic modulation, plasticity and learning in the dorsal striatum. Neuropharmacology, v.58, n.7, Jun, p.951-61. 2010.

Lukyanets, I. A. e E. A. Lukyanetz. Modulation of calcium signalling by the endoplasmic reticulum in Carassius neurons. Biochem Biophys Res Commun, Mar 22. 2013.

Lunkes, G. I., D. Lunkes*, et al.* Enzymes that hydrolyze adenine nucleotides in diabetes and associated pathologies. Thromb Res, v.109, n.4, Feb 15, p.189-94. 2003.

Lynch, M. A. Long-term potentiation and memory. Physiol Rev, v.84, n.1, Jan, p.87-136. 2004.

Macready, A. L., O. B. Kennedy*, et al.* Flavonoids and cognitive function: a review of human randomized controlled trial studies and recommendations for future studies. Genes Nutr, v.4, n.4, Dec, p.227-42. 2009.

Maggi, S., M. Noale*, et al.* Validity of the ATP III diagnostic criteria for the metabolic syndrome in an elderly Italian Caucasian population: the Italian Longitudinal Study on Aging. Atherosclerosis, v.197, n.2, Apr, p.877-82. 2008.

Maher, P. Modulation of multiple pathways involved in the maintenance of neuronal function during aging by fisetin. Genes Nutr, v.4, n.4, Dec, p.297-307. 2009.

Maher, P., T. Akaishi*, et al.* Flavonoid fisetin promotes ERK-dependent long-term potentiation and enhances memory. Proc Natl Acad Sci U S A, v.103, n.44, Oct 31, p.16568-73. 2006.

Maia, L. e A. De Mendonca. Does caffeine intake protect from Alzheimer's disease? Eur J Neurol, v.9, n.4, Jul, p.377-82. 2002.

Malatova, Z., A. Nicak*, et al.* Acetylcholinesterase activity and its molecular forms in selected regions of the dog's nervous system. Physiol Bohemoslov, v.29, n.5, p.415-22. 1980.

Mamede, M. E. O. e G. M. Pastore. Compostos Fenólicos do Vinho: Estrutura e Ação Antioxidante. Centro de Pesquisa e Processamento de Alimentos, v.22, n.3, p.233-252. 2004.

Manach, C., A. Scalbert*, et al.* Polyphenols: food sources and bioavailability. Am J Clin Nutr, v.79, n.5, May, p.727-47. 2004.

Mandairon, N., C. J. Ferretti*, et al.* Cholinergic modulation in the olfactory bulb influences spontaneous olfactory discrimination in adult rats. Eur J Neurosci, v.24, n.11, Dec, p.3234-44. 2006.

Mata, A. M., M. Berrocal*, et al.* Impairment of the activity of the plasma membrane Ca(2)(+)-ATPase in Alzheimer's disease. Biochem Soc Trans, v.39, n.3, Jun, p.819-22. 2011.

Mata, A. M. e M. R. Sepulveda. Plasma membrane Ca-ATPases in the nervous system during development and ageing. World J Biol Chem, v.1, n.7, Jul 26, p.229-34. 2010.

Matsui, T., I. A. Ogunwande*, et al.* Anti-hyperglycemic Potential of Natural Products. Mini Rev Med Chem, v.6, n.3, Mar, p.349-56. 2006.

Matsumoto, H., H. Inaba*, et al.* Orally administered delphinidin 3-rutinoside and cyanidin 3-rutinoside are directly absorbed in rats and humans and appear in the blood as the intact forms. J Agric Food Chem, v.49, n.3, Mar, p.1546-51. 2001.

Mazzanti, C. M., R. M. Spanevello*, et al.* Acetylcholinesterase activity in rats experimentally demyelinated with ethidium bromide and treated with interferon beta. Neurochem Res, v.31, n.8, Aug, p.1027-34. 2006.

Mcghie, T. K., G. D. Ainge*, et al.* Anthocyanin glycosides from berry fruit are absorbed and excreted unmetabolized by both humans and rats. J Agric Food Chem, v.51, n.16, Jul 30, p.4539-48. 2003.

Mehan, S., R. Arora*, et al.* Dementia – A Complete Literature Review on Various Mechanisms Involves in Pathogenesis and an Intracerebroventricular Streptozotocin Induced Alzheimer’s Disease. 2012.

Mehta, P. D. Amyloid beta protein as a marker or risk factor of Alzheimer's disease. Curr Alzheimer Res, v.4, n.4, Sep, p.359-63. 2007.

Messer, W. S., Jr., M. Bohnett*, et al.* Evidence for a preferential involvement of M1 muscarinic receptors in representational memory. Neurosci Lett, v.116, n.1-2, Aug 14, p.184-9. 1990.

Michaelis, E. K., M. L. Michaelis*, et al.* High affinity Ca2+-stimulated Mg2+-dependent ATPase in rat brain synaptosomes, synaptic membranes, and microsomes. J Biol Chem, v.258, n.10, May 25, p.6101-8. 1983.

Michon, A. The concept of mild cognitive impairment: relevance and limits in clinical practice. Front Neurol Neurosci, v.24, p.12-9. 2009.

Min, J., S. W. Yu*, et al.* Neuroprotective effect of cyanidin-3-O-glucoside anthocyanin in mice with focal cerebral ischemia. Neurosci Lett, v.500, n.3, Aug 18, p.157-61. 2011.

Miranda, K. M., M. G. Espey*, et al.* A rapid, simple spectrophotometric method for simultaneous detection of nitrate and nitrite. Nitric Oxide, v.5, n.1, Feb, p.62-71. 2001.

Missiaen, L., G. Callewaert*, et al.* [Intracellular calcium: physiology and physiopathology]. Verh K Acad Geneeskd Belg, v.62, n.6, p.471-99. 2000.

Missiaen, L., W. Robberecht*, et al.* Abnormal intracellular ca(2+)homeostasis and disease. Cell Calcium, v.28, n.1, Jul, p.1-21. 2000.

Miyazawa, T., K. Nakagawa*, et al.* Direct intestinal absorption of red fruit anthocyanins, cyanidin-3-glucoside and cyanidin-3,5-diglucoside, into rats and humans. J Agric Food Chem, v.47, n.3, Mar, p.1083-91. 1999.

Moseley, A. E., M. T. Williams*, et al.* Deficiency in Na,K-ATPase alpha isoform genes alters spatial learning, motor activity, and anxiety in mice. J Neurosci, v.27, n.3, Jan 17, p.616-26. 2007.

Mudnic, I., D. Budimir*, et al.* Antioxidant and Vasodilatory Effects of Blackberry and Grape Wines. J Med Food, Nov 14. 2011.

Muller, D., R. M. Nitsch*, et al.* Streptozotocin increases free fatty acids and decreases phospholipids in rat brain. J Neural Transm, v.105, n.10-12, p.1271-81. 1998.

Musial, A., M. Bajda*, et al.* Recent developments in cholinesterases inhibitors for Alzheimer's disease treatment. Curr Med Chem, v.14, n.25, p.2654-79. 2007.

Nagy, A. e A. V. Delgado-Escueta. Rapid preparation of synaptosomes from mammalian brain using nontoxic isoosmotic gradient material (Percoll). J Neurochem, v.43, n.4, Oct, p.1114-23. 1984.

Nguyen, M. L., G. D. Cox*, et al.* Kinetic parameters for the vesicular acetylcholine transporter: two protons are exchanged for one acetylcholine. Biochemistry, v.37, n.38, Sep 22, p.13400-10. 1998.

Nishizuka, T., Y. Fujita*, et al.* Procyanidins are potent inhibitors of LOX-1: a new player in the French Paradox. Proc Jpn Acad Ser B Phys Biol Sci, v.87, n.3, p.104-13. 2011.

Oh, S. R., S. J. Kim*, et al.* Angelica keiskei ameliorates scopolamine-induced memory impairments in mice. Biol Pharm Bull, v.36, n.1, p.82-8. 2013.

Paleari, L., A. Grozio*, et al.* The cholinergic system and cancer. Semin Cancer Biol, v.18, n.3, Jun, p.211-7. 2008.

Panayiotidis, M. I., R. Franco*, et al.* Ouabain-induced perturbations in intracellular ionic homeostasis regulate death receptor-mediated apoptosis. Apoptosis, v.15, n.7, Jul, p.834-49. 2010.

Parsons, J. T., D. A. Sun*, et al.* Neuronal-specific endoplasmic reticulum Mg(2+)/Ca(2+) ATPase Ca(2+) sequestration in mixed primary hippocampal culture homogenates. Anal Biochem, v.330, n.1, Jul 1, p.130-9. 2004.

Passamonti, S., U. Vrhovsek*, et al.* The stomach as a site for anthocyanins absorption from food. FEBS Lett, v.544, n.1-3, Jun 5, p.210-3. 2003.

_____. Fast access of some grape pigments to the brain. J Agric Food Chem, v.53, n.18, Sep 7, p.7029-34. 2005.

Pathan, A. R., B. Viswanad*, et al.* Chronic administration of pioglitazone attenuates intracerebroventricular streptozotocin induced-memory impairment in rats. Life Sci, v.79, n.23, Nov 2, p.2209-16. 2006.

Paula-Lima, A. C., F. G. De Felice*, et al.* Activation of GABA(A) receptors by taurine and muscimol blocks the neurotoxicity of beta-amyloid in rat hippocampal and cortical neurons. Neuropharmacology, v.49, n.8, Dec, p.1140-8. 2005.

Pavlov, K. V. e V. S. Sokolov. Electrogenic ion transport by Na+,K+-ATPase. Membr Cell Biol, v.13, n.6, p.745-88. 2000.

Paxinos, G. e C. Watson. The Rat Brain in Stereotaxic Coordinates. Academic Press, San Diego. 1986.

Pereira, C., C. Ferreira*, et al.* Contribution of plasma membrane and endoplasmic reticulum Ca(2+)-ATPases to the synaptosomal [Ca2+]i increase during oxidative stress. Brain Res, v.713, n.1-2, Mar 25, p.269-77. 1996.

Pergola, C., A. Rossi*, et al.* Inhibition of nitric oxide biosynthesis by anthocyanin fraction of blackberry extract. Nitric Oxide, v.15, n.1, Aug, p.30-9. 2006.

Pinton, S., J. T. Da Rocha*, et al.* Sporadic dementia of Alzheimer's type induced by streptozotocin promotes anxiogenic behavior in mice. Behav Brain Res, v.223, n.1, Sep 30, p.1-6. 2011.

Pinton, S., J. T. Da Rocha*, et al.* Organoselenium improves memory decline in mice: involvement of acetylcholinesterase activity. Neurosci Lett, v.472, n.1, Mar 12, p.56-60. 2010.

Prast, H. e A. Philippu. Nitric oxide as modulator of neuronal function. Prog Neurobiol, v.64, n.1, May, p.51-68. 2001.

Puma, C., O. Deschaux*, et al.* Nicotine improves memory in an object recognition task in rats. Eur Neuropsychopharmacol, v.9, n.4, Jun, p.323-7. 1999.

Raeymaekers, L. e F. Wuytack. [The Ca(2+)-transport ATPases of smooth muscle]. Verh K Acad Geneeskd Belg, v.53, n.6, p.605-28. 1991.

Ramirez, M. R., I. Izquierdo*, et al.* Effect of lyophilised Vaccinium berries on memory, anxiety and locomotion in adult rats. Pharmacol Res, v.52, n.6, Dec, p.457-62. 2005.

Rand, J. B. Acetylcholine. WormBook, p.1-21. 2007.

Rang, H. P., M. M. Dale*, et al.* Cholinergic transmission. In: Pharmacology. Edinburgh, UK: Harcourt Publishers Ltd,, v.4, p.110-138. 2001.

Rang Oh, S., S. Jin Kim*, et al.* Angelica keiskei ameliorates scopolamine-induced memory impairments in mice. Biol Pharm Bull, Nov 5. 2012.

Ray, C. A., J. Blin*, et al.* Effects of cholinergic agonists on regional brain energy metabolism in the scopolamine-treated rat. Neuropharmacology, v.31, n.11, Nov, p.1193-9. 1992.

Rechner, A. R., M. A. Smith*, et al.* Colonic metabolism of dietary polyphenols: influence of structure on microbial fermentation products. Free Radic Biol Med, v.36, n.2, Jan 15, p.212-25. 2004.

Reed, T. T., W. M. Pierce*, et al.* Proteomic identification of HNE-bound proteins in early Alzheimer disease: Insights into the role of lipid peroxidation in the progression of AD. Brain Res, v.1274, Jun 5, p.66-76. 2009.

Rein, M. Copigmentation reactions and color stability of berry anthocyanins. Helsinki: University of Helsinki, v.1, p.10-14. 2005.

Rendeiro, C., J. P. Spencer*, et al.* The impact of flavonoids on spatial memory in rodents: from behaviour to underlying hippocampal mechanisms. Genes Nutr, v.4, n.4, Dec, p.251-70. 2009.

Rendeiro, C., D. Vauzour*, et al.* Blueberry supplementation induces spatial memory improvements and region-specific regulation of hippocampal BDNF mRNA expression in young rats. Psychopharmacology (Berl), v.223, n.3, Oct, p.319-30. 2012.

Rizopoulos, E., J. P. Chambers*, et al.* Kinetic properties of the [Ca2+ + Mg2+]-ATPase in Alzheimer and normal fibroblasts at low free calcium. Brain Res Bull, v.21, n.5, Nov, p.825-8. 1988.

Rizopoulos, E., J. P. Chambers*, et al.* Effects of free Ca2+ on the [Ca2+ + Mg2+]-dependent adenosinetriphosphatase (ATPase) of Alzheimer and normal fibroblasts. Neurobiol Aging, v.10, n.6, Nov-Dec, p.717-20. 1989.

Robson, S. C., J. Sevigny*, et al.* The E-NTPDase family of ectonucleotidases: Structure function relationships and pathophysiological significance. Purinergic Signal, v.2, n.2, Jun, p.409-30. 2006.

Rocha, J. B., T. Emanuelli*, et al.* Effects of early undernutrition on kinetic parameters of brain acetylcholinesterase from adult rats. Acta Neurobiol Exp (Wars), v.53, n.3, p.431-7. 1993.

Rogers, J. L. e R. P. Kesner. Cholinergic modulation of the hippocampus during encoding and retrieval of tone/shock-induced fear conditioning. Learn Mem, v.11, n.1, Jan-Feb, p.102-7. 2004.

Rogers, L. J., R. Oettinger*, et al.* Separate chemical inhibitors of long-term and short-term memory: contrasting effects of cycloheximide, ouabain and ethacrynic acid on various learning tasks in chickens. Proc R Soc Lond B Biol Sci, v.196, n.1123, Mar 4, p.171-95. 1977.

Rohn, T. T., T. R. Hinds*, et al.* Ion transport ATPases as targets for free radical damage. Protection by an aminosteroid of the Ca2+ pump ATPase and Na+/K+ pump ATPase of human red blood cell membranes. Biochem Pharmacol, v.46, n.3, Aug 3, p.525-34. 1993.

Rose, A. M. e R. Valdes, Jr. Understanding the sodium pump and its relevance to disease. Clin Chem, v.40, n.9, Sep, p.1674-85. 1994.

Rubin, M. A., C. A. Albach*, et al.* Anxiolytic-like effects of 4-phenyl-2-trichloromethyl-3H-1, 5-benzodiazepine hydrogen sulfate in mice. Braz J Med Biol Res, v.33, n.9, Sep, p.1069-73. 2000.

Rubin, M. A., R. L. Boemo*, et al.* Intrahippocampal spermidine administration improves inhibitory avoidance performance in rats. Behav Pharmacol, v.11, n.1, Feb, p.57-61. 2000.

Ruknudin, A. M. e E. G. Lakatta. The regulation of the Na/Ca exchanger and plasmalemmal Ca2+ ATPase by other proteins. Ann N Y Acad Sci, v.1099, Mar, p.86-102. 2007.

Rutkowski, R., S. A. Pancewicz*, et al.* [Reactive oxygen and nitrogen species in inflammatory process]. Pol Merkur Lekarski, v.23, n.134, Aug, p.131-6. 2007.

Saija, A., P. Princi*, et al.* Effect of Vaccinium myrtillus anthocyanins on triiodothyronine transport into brain in the rat. Pharmacol Res, v.22 Suppl 3, Sep-Oct, p.59-60. 1990.

Sakurai, T., T. Kato*, et al.* Nefiracetam elevates extracellular acetylcholine level in the frontal cortex of rats with cerebral cholinergic dysfunctions: an in vivo microdialysis study. Neurosci Lett, v.246, n.2, Apr 24, p.69-72. 1998.

Salkovic-Petrisic, M. Amyloid cascade hypothesis: is it true for sporadic Alzheimer's disease. Periodicum Biologorum, v.110, n.1, p.17-25. 2008.

Salthouse, T. A. Memory aging from 18 to 80. Alzheimer Disease Association Disorder, v.17, p.162-167. 2003.

Sambamurti, K., N. H. Greig*, et al.* Advances in the cellular and molecular biology of the beta-amyloid protein in Alzheimer's disease. Neuromolecular Med, v.1, n.1, p.1-31. 2002.

Sansoni, P., R. Vescovini*, et al.* The immune system in extreme longevity. Exp Gerontol, v.43, n.2, Feb, p.61-5. 2008.

Sato, T., K. Tanaka*, et al.* Effects of steroid hormones on (Na+, K+)-ATPase activity inhibition-induced amnesia on the step-through passive avoidance task in gonadectomized mice. Pharmacol Res, v.49, n.2, Feb, p.151-9. 2004.

Sattler, R., Z. Xiong*, et al.* Specific coupling of NMDA receptor activation to nitric oxide neurotoxicity by PSD-95 protein. Science, v.284, n.5421, Jun 11, p.1845-8. 1999.

Scalbert, A. e G. Williamson. Dietary intake and bioavailability of polyphenols. J Nutr, v.130, n.8S Suppl, Aug, p.2073S-85S. 2000.

Scarpini, E. e F. Cogiamanian. Alzheimer's disease: from molecular pathogenesis to innovative therapies. Expert Rev Neurother, v.3, n.5, Sep, p.619-30. 2003.

Scarpini, E., P. Scheltens*, et al.* Treatment of Alzheimer's disease: current status and new perspectives. Lancet Neurol, v.2, n.9, Sep, p.539-47. 2003.

Schafer, M. K., L. E. Eiden*, et al.* Cholinergic neurons and terminal fields revealed by immunohistochemistry for the vesicular acetylcholine transporter. II. The peripheral nervous system. Neuroscience, v.84, n.2, May, p.361-76. 1998.

Schetinger, M. R., C. K. Barcellos*, et al.* Activity of synaptosomal ATP diphosphohydrolase from hippocampus of rats tolerant to forebrain ischemia. Braz J Med Biol Res, v.27, n.5, May, p.1123-8. 1994.

Schetinger, M. R., V. M. Morsch*, et al.* NTPDase and 5'-nucleotidase activities in physiological and disease conditions: new perspectives for human health. Biofactors, v.31, n.2, p.77-98. 2007.

Schetinger, M. R., V. L. Vieira*, et al.* ATP and ADP hydrolysis in fish, chicken and rat synaptosomes. Comp Biochem Physiol B Biochem Mol Biol, v.128, n.4, Apr, p.731-41. 2001.

Schmatz, R., C. M. Mazzanti*, et al.* Ectonucleotidase and acetylcholinesterase activities in synaptosomes from the cerebral cortex of streptozotocin-induced diabetic rats and treated with resveratrol. Brain Res Bull, v.80, n.6, Dec 16, p.371-6. 2009.

Schmatz, R., M. R. Schetinger*, et al.* Effects of resveratrol on nucleotide degrading enzymes in streptozotocin-induced diabetic rats. Life Sci, v.84, n.11-12, Mar 13, p.345-50. 2009.

Schmued, L. C., C. C. Stowers*, et al.* Fluoro-Jade C results in ultra high resolution and contrast labeling of degenerating neurons. Brain Res, v.1035, n.1, Feb 21, p.24-31. 2005.

Scuri, R., P. Lombardo*, et al.* Inhibition of Na+/K+ ATPase potentiates synaptic transmission in tactile sensory neurons of the leech. Eur J Neurosci, v.25, n.1, Jan, p.159-67. 2007.

Sharma, M. e Y. K. Gupta. Effect of chronic treatment of melatonin on learning, memory and oxidative deficiencies induced by intracerebroventricular streptozotocin in rats. Pharmacol Biochem Behav, v.70, n.2-3, Oct-Nov, p.325-31. 2001a.

_____. Intracerebroventricular injection of streptozotocin in rats produces both oxidative stress in the brain and cognitive impairment. Life Sci, v.68, n.9, Jan 19, p.1021-9. 2001b.

Shen, Z. X. Brain cholinesterases: I. The clinico-histopathological and biochemical basis of Alzheimer's disease. Med Hypotheses, v.63, n.2, p.285-97. 2004.

Shibata, K., Y. Shibata*, et al.* Organic Chemistry. J. Am. Chem. Soc v.41, p.208-220. 1912.

Shih, P. H., Y. C. Chan*, et al.* Antioxidant and cognitive promotion effects of anthocyanin-rich mulberry (Morus atropurpurea L.) on senescence-accelerated mice and prevention of Alzheimer's disease. J Nutr Biochem, v.21, n.7, Jul, p.598-605. 2010.

Shimmyo, Y., T. Kihara*, et al.* Flavonols and flavones as BACE-1 inhibitors: structure-activity relationship in cell-free, cell-based and in silico studies reveal novel pharmacophore features. Biochim Biophys Acta, v.1780, n.5, May, p.819-25. 2008.

Shin, W. H., S. J. Park*, et al.* Protective effect of anthocyanins in middle cerebral artery occlusion and reperfusion model of cerebral ischemia in rats. Life Sci, v.79, n.2, Jun 6, p.130-7. 2006.

Shukitt-Hale, B., V. Cheng*, et al.* Effects of blackberries on motor and cognitive function in aged rats. Nutr Neurosci, v.12, n.3, Jun, p.135-40. 2009.

Skou, J. C. The influence of some cations on an adenosine triphosphatase from peripheral nerves. Biochim Biophys Acta, v.23, n.2, Feb, p.394-401. 1957.

Skou, J. C. e M. Esmann. The Na,K-ATPase. J Bioenerg Biomembr, v.24, n.3, Jun, p.249-61. 1992.

Sonkusare, S., K. Srinivasan*, et al.* Effect of donepezil and lercanidipine on memory impairment induced by intracerebroventricular streptozotocin in rats. Life Sci, v.77, n.1, May 20, p.1-14. 2005.

Soubelet, A. e T. A. Salthouse. Correlates of level and change in the Mini-Mental State Examination. Psychol Assess, v.23, n.4, Dec, p.811-8. 2011.

Southam, E., P. K. Thomas*, et al.* Experimental vitamin E deficiency in rats. Morphological and functional evidence of abnormal axonal transport secondary to free radical damage. Brain, v.114 (Pt 2), Apr, p.915-36. 1991.

Spanevello, R. M., C. M. Mazzanti*, et al.* Apyrase and 5'-nucleotidase activities in synaptosomes from the cerebral cortex of rats experimentally demyelinated with ethidium bromide and treated with interferon-beta. Neurochem Res, v.31, n.4, Apr, p.455-62. 2006.

Spencer, J. P. Nutrients and brain health: an overview. Genes Nutr, v.4, n.4, Dec, p.225-6. 2009.

_____. The impact of fruit flavonoids on memory and cognition. Br J Nutr, v.104 Suppl 3, Oct, p.S40-7. 2010.

Stone, W. S., B. Walser*, et al.* Scopolamine- and morphine-induced impairments of spontaneous alternation performance in mice: reversal with glucose and with cholinergic and adrenergic agonists. Behav Neurosci, v.105, n.2, Apr, p.264-71. 1991.

Swerdlow, R. H. Is aging part of Alzheimer's disease, or is Alzheimer's disease part of aging? Neurobiol Aging, v.28, n.10, Oct, p.1465-80. 2007.

_____. Brain aging, Alzheimer's disease, and mitochondria. Biochim Biophys Acta, v.1812, n.12, Dec, p.1630-9. 2011.

Takeguchi, C. A., U. E. Honegger*, et al.* Evidence for subclasses of SH groups in (Na++K+)-ATPase. Life Sci, v.19, n.6, Sep 15, p.797-805. 1976.

Talavera, S., C. Felgines*, et al.* Anthocyanin metabolism in rats and their distribution to digestive area, kidney, and brain. J Agric Food Chem, v.53, n.10, May 18, p.3902-8. 2005.

Talavera, S., C. Felgines*, et al.* Anthocyanins are efficiently absorbed from the stomach in anesthetized rats. J Nutr, v.133, n.12, Dec, p.4178-82. 2003.

Talavera, S., C. Felgines*, et al.* Anthocyanins are efficiently absorbed from the small intestine in rats. J Nutr, v.134, n.9, Sep, p.2275-9. 2004.

Tang, Y., M. Mishkin*, et al.* Effects of muscarinic blockade in perirhinal cortex during visual recognition. Proc Natl Acad Sci U S A, v.94, n.23, Nov 11, p.12667-9. 1997.

Tarozzi, A., A. Marchesi*, et al.* Protective effects of cyanidin-3-O-beta-glucopyranoside against UVA-induced oxidative stress in human keratinocytes. Photochem Photobiol, v.81, n.3, May-Jun, p.623-9. 2005.

Tarozzi, A., A. Merlicco*, et al.* Cyanidin 3-O-glucopyranoside protects and rescues SH-SY5Y cells against amyloid-beta peptide-induced toxicity. Neuroreport, v.19, n.15, Oct 8, p.1483-6. 2008.

Tarozzi, A., F. Morroni*, et al.* Neuroprotective effects of anthocyanins and their in vivo metabolites in SH-SY5Y cells. Neurosci Lett, v.424, n.1, Aug 31, p.36-40. 2007.

Tarozzi, A., F. Morroni*, et al.* Neuroprotective effects of cyanidin 3-O-glucopyranoside on amyloid beta (25-35) oligomer-induced toxicity. Neurosci Lett, v.473, n.2, Apr 5, p.72-6. 2010.

Therien, A. G. e R. Blostein. Mechanisms of sodium pump regulation. Am J Physiol Cell Physiol, v.279, n.3, Sep, p.C541-66. 2000.

Timberlake, C. F. e P. Bridle. Interactions between anthocyanins, phenolic compounds, and acetaldehyde and their significance in red wines. American Journal of Enology and Viticulture v.27, p.97–105. 1976.

Tiwari, V., A. Kuhad*, et al.* Chronic treatment with tocotrienol, an isoform of vitamin E, prevents intracerebroventricular streptozotocin-induced cognitive impairment and oxidative-nitrosative stress in rats. Pharmacol Biochem Behav, v.93, n.2, Aug, p.183-9. 2009.

Tota, S., H. Awasthi*, et al.* Protective effect of quercetin against intracerebral streptozotocin induced reduction in cerebral blood flow and impairment of memory in mice. Behav Brain Res, v.209, n.1, May 1, p.73-9. 2010.

Tota, S., P. K. Kamat*, et al.* Candesartan improves memory decline in mice: involvement of AT1 receptors in memory deficit induced by intracerebral streptozotocin. Behav Brain Res, v.199, n.2, May 16, p.235-40. 2009.

Trevisan, G., G. Maldaner*, et al.* Antinociceptive effects of 14-membered cyclopeptide alkaloids. J Nat Prod, v.72, n.4, Apr, p.608-12. 2009.

Tsuda, T., F. Horio*, et al.* Absorption and metabolism of cyanidin 3-O-beta-D-glucoside in rats. FEBS Lett, v.449, n.2-3, Apr 23, p.179-82. 1999.

Vaillend, C., S. E. Mason*, et al.* Mechanisms of neuronal hyperexcitability caused by partial inhibition of Na+-K+-ATPases in the rat CA1 hippocampal region. J Neurophysiol, v.88, n.6, Dec, p.2963-78. 2002.

Valko, M., D. Leibfritz*, et al.* Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol, v.39, n.1, p.44-84. 2007.

Varadinova, M. G., D. I. Docheva-Drenska*, et al.* Effects of anthocyanins on learning and memory of ovariectomized rats. Menopause, v.16, n.2, Mar-Apr, p.345-9. 2009.

Veitch, N. C. e R. J. Grayer. Flavonoids and their glycosides, including anthocyanins. Nat Prod Rep, v.25, n.3, Jun, p.555-611. 2008.

_____. Flavonoids and their glycosides, including anthocyanins. Nat Prod Rep, v.28, n.10, Oct, p.1626-95. 2011.

Veldhuis, W. B., M. Van Der Stelt*, et al.* In vivo excitotoxicity induced by ouabain, a Na+/K+-ATPase inhibitor. J Cereb Blood Flow Metab, v.23, n.1, Jan, p.62-74. 2003.

Verkhratsky, A., J. J. Rodriguez*, et al.* Calcium signalling in astroglia. Mol Cell Endocrinol, v.353, n.1-2, Apr 28, p.45-56. 2012.

Vignini, A., L. Nanetti*, et al.* Modifications of platelet from Alzheimer disease patients: a possible relation between membrane properties and NO metabolites. Neurobiol Aging, v.28, n.7, Jul, p.987-94. 2007.

Vogel, R. A., G. D. Frye*, et al.* Attenuation of the effects of punishment by ethanol: comparisons with chlordiazepoxide. Psychopharmacology (Berl), v.71, n.2, p.123-9. 1980.

Volonte, C., S. Amadio*, et al.* Receptor webs: can the chunking theory tell us more about it? Brain Res Rev, v.59, n.1, Nov, p.1-8. 2008.

Wang, H., Y. Lu*, et al.* Differentiating effects of anisodamine on cognitive amelioration and peripheral muscarinic side effects induced by pilocarpine in mice. Neurosci Lett, v.344, n.3, Jul 3, p.173-6. 2003.

Wang, L. S. e G. D. Stoner. Anthocyanins and their role in cancer prevention. Cancer Lett, v.269, n.2, Oct 8, p.281-90. 2008.

Wang, Q., M. Xia*, et al.* Cyanidin-3-O-beta-glucoside inhibits iNOS and COX-2 expression by inducing liver X receptor alpha activation in THP-1 macrophages. Life Sci, v.83, n.5-6, Aug 1, p.176-84. 2008.

Waterhouse, A. L. Wine phenolics. Ann N Y Acad Sci, v.957, May, p.21-36. 2002.

Wesnes, K. A., P. M. Simpson*, et al.* Cholinesterase inhibition in the scopolamine model of dementia. Ann N Y Acad Sci, v.640, p.268-71. 1991.

Wharton, S. B., J. P. O'callaghan*, et al.* Population variation in glial fibrillary acidic protein levels in brain ageing: relationship to Alzheimer-type pathology and dementia. Dement Geriatr Cogn Disord, v.27, n.5, p.465-73. 2009.

Whiting, D. A. Natural phenolic compounds 1900-2000: a bird's eye view of a century's chemistry. Nat Prod Rep, v.18, n.6, Dec, p.583-606. 2001.

Wieraszko, A. Extracellular ATP as a neurotransmitter: its role in synaptic plasticity in the hippocampus. Acta Neurobiol Exp (Wars), v.56, n.2, p.637-48. 1996.

Wieraszko, A. e Y. H. Ehrlich. On the role of extracellular ATP in the induction of long-term potentiation in the hippocampus. J Neurochem, v.63, n.5, Nov, p.1731-8. 1994.

Williams, C. A. e R. J. Grayer. Anthocyanins and other flavonoids. Nat Prod Rep, v.21, n.4, Aug, p.539-73. 2004.

Williams, C. M., M. A. El Mohsen*, et al.* Blueberry-induced changes in spatial working memory correlate with changes in hippocampal CREB phosphorylation and brain-derived neurotrophic factor (BDNF) levels. Free Radic Biol Med, v.45, n.3, Aug 1, p.295-305. 2008.

Williams, R. J. e J. P. Spencer. Flavonoids, cognition, and dementia: actions, mechanisms, and potential therapeutic utility for Alzheimer disease. Free Radic Biol Med, v.52, n.1, Jan 1, p.35-45. 2012.

Winters, B. D. e T. J. Bussey. Removal of cholinergic input to perirhinal cortex disrupts object recognition but not spatial working memory in the rat. Eur J Neurosci, v.21, n.8, Apr, p.2263-70. 2005.

Woodhouse, A., C. E. Shepherd*, et al.* Cytoskeletal alterations differentiate presenilin-1 and sporadic Alzheimer's disease. Acta Neuropathol, v.117, n.1, Jan, p.19-29. 2009.

Wortmann, M. Dementia: a global health priority - highlights from an ADI and World Health Organization report. Alzheimers Res Ther, v.4, n.5, Sep 21, p.40. 2012.

Wrolstad, R. E., R. W. Durst*, et al.* Tracking color and pigment changes in anthocyanin products. Trends in Food Science & Technology v.16, p.423-428. 2005.

Wu, X. e R. L. Prior. Identification and characterization of anthocyanins by High-performance liquid chromatography–electrospray ionization–tandem mass spectrometry in common foods in the United States: Vegetables, nuts, and grains. Journal of Agricultural and Food Chemistry, v.53, n.8, p.3101-3113. 2005.

Wyse, A. T., C. S. Bavaresco*, et al.* Nitric oxide synthase inhibition by L-NAME prevents the decrease of Na+,K+-ATPase activity in midbrain of rats subjected to arginine administration. Neurochem Res, v.26, n.5, May, p.515-20. 2001.

Wyse, A. T., C. S. Bavaresco*, et al.* Training in inhibitory avoidance causes a reduction of Na+,K+-ATPase activity in rat hippocampus. Physiol Behav, v.80, n.4, Jan, p.475-9. 2004.

Wyse, A. T., E. L. Streck*, et al.* Methylmalonate administration decreases Na+,K+-ATPase activity in cerebral cortex of rats. Neuroreport, v.11, n.10, Jul 14, p.2331-4. 2000.

Xiao, A. Y., L. Wei*, et al.* Ionic mechanism of ouabain-induced concurrent apoptosis and necrosis in individual cultured cortical neurons. J Neurosci, v.22, n.4, Feb 15, p.1350-62. 2002.

Yamaguchi, M. Role of regucalcin in brain calcium signaling: involvement in aging. Integr Biol (Camb), v.4, n.8, Aug, p.825-37. 2012.

Yamashima, T. Reconsider Alzheimer's disease by the 'calpain-cathepsin hypothesis'-A perspective review. Prog Neurobiol, Mar 15. 2013.

Yoshida, K., M. Mori*, et al.* Blue flower color development by anthocyanins: from chemical structure to cell physiology. Nat Prod Rep, v.26, n.7, Jul, p.884-915. 2009.

Youdim, K. A., M. S. Dobbie*, et al.* Interaction between flavonoids and the blood-brain barrier: in vitro studies. J Neurochem, v.85, n.1, Apr, p.180-92. 2003.

Zaidi, A. Plasma membrane Ca-ATPases: Targets of oxidative stress in brain aging and neurodegeneration. World J Biol Chem, v.1, n.9, Sep 26, p.271-80. 2010.

Zakut, H., A. Matzkel*, et al.* Polymorphism of acetylcholinesterase in discrete regions of the developing human fetal brain. J Neurochem, v.45, n.2, Aug, p.382-9. 1985.

Zawia, N. H., D. K. Lahiri*, et al.* Epigenetics, oxidative stress, and Alzheimer disease. Free Radic Biol Med, v.46, n.9, May 1, p.1241-9. 2009.

Zhan, H., T. Tada*, et al.* Spatial learning transiently disturbed by intraventricular administration of ouabain. Neurol Res, v.26, n.1, Jan, p.35-40. 2004.

Zhang, L., F. Guo*, et al.* Na(+) /K(+) -ATPase inhibition upregulates NMDA-evoked currents in rat hippocampal CA1 pyramidal neurons. Fundam Clin Pharmacol, Apr 26. 2011.

_____. Na(+)/K(+)-ATPase inhibition upregulates NMDA-evoked currents in rat hippocampal CA1 pyramidal neurons. Fundam Clin Pharmacol, v.26, n.4, Aug, p.503-12. 2012.

Zilkens, R. R., W. A. Davis*, et al.* Earlier Age of Dementia Onset and Shorter Survival Times in Dementia Patients With Diabetes. Am J Epidemiol, Mar 28. 2013.

Zimmermann, H. Signalling via ATP in the nervous system. Trends Neurosci, v.17, n.10, Oct, p.420-6. 1994.

_____. BIOCHEMISTRY, LOCALIZATION AND FUNCTIONAL ROLES OF ECTO-NUCLEOTIDASES IN THE NERVOUS SYSTEM. Progress in Neurobiology, v.49, n.6, p.589-618. 1996.

_____. Ectonucleotidases: some recent developments and a note on nomenclature. Drug Development Res, v.52, n.44-56. 2001.

Zimmermann, H., N. Braun*, et al.* New insights into molecular structure and function of ectonucleotidases in the nervous system. Neurochem Int, v.32, n.5-6, p.421-425. 1998.

Zini, A., D. Del Rio*, et al.* Do flavan-3-ols from green tea reach the human brain? Nutr Neurosci, v.9, n.1-2, Feb-Apr, p.57-61. 2006.