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

Michael da Costa

EFEITO DA N-ACETILCISTEÍNA NO DÉFICIT COGNITIVO INDUZIDO PELA ESTREPTOZOTOCINA EM CAMUNDONGOS

Santa Maria, RS
2016

Michael da Costa

EFEITO DA N-ACETILCISTEÍNA NO DÉFICIT COGNITIVO INDUZIDO PELA ESTREPTOZOTOCINA EM CAMUNDONGOS

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

Orientadora: Profª. Drª. Maria E. Pereira
Co-orientador: Prof. Dr. Ricardo Brandão
Co-orientadora: Profª. Drª. Cecília M.P. Rodrigues

Santa Maria, RS, Brasil
2016

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

da Costa, Michael
EFEITO DA N-ACETILCISTEÍNA NO DÉFICIT COGNITIVO INDUZIDO PELA ESTREPTOZOTOCINA EM CAMUNDONGOS / Michael da Costa.- 2016.
126 p.; 30 cm

Orientador: Maria Ester Pereira
Coorientador: Ricardo Brandão
Tese (doutorado) - Universidade Federal de Santa Maria, Centro de Ciências Naturais e Exatas, Programa de Pós-Graduação em Bioquímica Toxicológica, RS, 2016

1. Cognição 2. Alzheimer 3. Colinesterase 4. Anti-colinesterásicos 5. Neuroproteção I. Pereira, Maria Ester II. Brandão, Ricardo III. Título.

Michael da Costa

EFEITO DA N-ACETILCISTEÍNA NO DÉFICIT COGNITIVO INDUZIDO PELA ESTREPTOZOTOCINA EM CAMUNDONGOS

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

Aprovado em 15 de Julho de 2016:

_____

Maria Ester Pereira, PhD. (UFSM)
(Presidente/Orientador)

_____

Carmen Juracy Silveira Gottfried, PhD. (UFRGS)

_____

Adair Roberto Soares dos Santos, Dr. (UFSC)

_____

Gustavo Orione Puntel, Dr. (UFSM)

_____

Denis Broock Rosemberg, Dr. (UFSM)

Santa Maria, RS
2016

DEDICATÓRIA

… para Arlindo e Elizabeth.

AGRADECIMENTOS

À minha mãe **Elizabeth**, por ter sempre acreditado em mim, por ser sempre a primeira em me apoiar e principalmente por ser o maior exemplo de fortaleza que tenho.

Ao meu pai **Arlindo** por ter sempre visto o melhor em mim.

Ao meu irmão **Edgar** e minhas irmãs **Karen** e **Fernanda**, pela compreensão e carinho que sempre tiveram comigo e para mim.

Aos meus sobrinhos **Yian**, **Tais** e **Pedro**, por terem sido sempre o principal motivo para eu acreditar e querer seguir em frente.

À minha avó **Irma** pelo exemplo de ser humano e perseverança.

Ao meu padrasto **Algenor**, cunhado **Leandro** e cunhada **Ana**, por terem sempre me apoiado.

A todos estes por fazerem de mim uma pessoa melhor.

Aos meus colegas de laboratório **Taise**, **Mariana**, **Lidiane, Cláudia** e **Rafael** pelo companheirismo e pela amizade.

À minha orientadora, professora **Ester**, por ter possibilitado a realização deste doutorado e a professora **Cristina** quem me deu a chance de realizar o mestrado. Ao meu co-orientador **Ricardo** (mais que um orientador, um verdadeiro amigo) e co-orientadora **Cecília**. Estes todos, exemplos de professionais. Muito obrigado pelo aprendizado, dedicação, compreensão e carinho.

Aos meus amigos **Tiago, Vitor, Tiagão, André, Alisson, César, Anderson, Helton, Renan, Filipe, Juliano, Benhur, Marlon** e **Matheus**, pela parceria, futebol, churrascos, cervejas, conversas, entre outras coisas. Mas principalmente, pela amizade e pelo aprendizado.

Às minhas amigas **Simone**, **Carmine**, **Tuane**, **Adriane**, **Mayara**, **Caroline** e **Jamile** pela amizade e por sempre estarem presentes quando precisei.

Aos meus colegas do iMed**, Maria, Tânia, André, Pedro, André, Joana, Miguel, Sofia, Maria, Hugo, Dionísio, Rui** e **Diane**, pelo maravilhoso ano que foi ao ter trabalhado junto a vocês.

À Universidade Federal de Santa Maria

À Universidade de Lisboa

Ao PPGBTox

Ao 18

RESUMO

EFEITO DA N-ACETILCISTEÍNA NO DÉFICIT COGNITIVO INDUZIDO PELA ESTREPTOZOTOCINA EM CAMUNDONGO

AUTOR: Michael da Costa
ORIENTADORA: Maria E. Pereira
CO-ORIENTADOR: Ricardo Brandão
CO-ORIENTADORA: Cecília M.P. Rodrigues

O déficit cognitivo é uma desordem mental que está associado a doenças neurodegenerativas, como é a doença de Alzheimer (DA) a qual é a forma mais comum de demência. Atualmente não existem evidências consistentes quem permitam apoiar qualquer medida para a prevenção desta doença (DAVIGLUS e col., 2010). Já para o seu tratamento, existem métodos os quais proporcionam alívio relativo dos sintomas, contudo, de natureza paliativa[1]. Assim, o presente trabalho visou avaliar a n-acetilcisteína (NAC), uma molécula com propriedades neuroprotetora, no tratamento e prevenção da demência consequente da DA, utilizando-se de um modelo experimental de demência induzida pela estreptozotocina (STZ) em camundongos. Inicialmente avaliou-se o efeito da NAC sobre a atividade da acetilcolinesterase (AChE) cerebral de camundongos *in vitro*. A concentração de ditiobisnitrobenzoato (DTNB) e pH ideais foi de 0,3 mM e 7,4, respectivamente. Obteve-se linearidade na atividade enzimática com concentrações de 0,025 a 0,450 mM de acetiltiocolina (ATCh). Foram adicionados 60 sec de intervalo prévios à adição da ATCh para evitar a interferência da interação DTNB-NAC. A NAC interferiu na V_{max} da AChE a partir da concentração de 75 µM sem modificar a K_m, caracterizando uma inibição de forma não-competitiva. Em uma segunda instância, avaliou-se o efeito da NAC sobre o déficit cognitivo a curto prazo em camundongos. Para isso, os animais foram divididos em quatro grupos e foram tratados com NAC (50 mg/kg/dia v.o.) ou salina por nove dias consecutivos e com STZ (2.5 mg/kg i.c.v.) ou FCEa no primeiro e terceiro dias. Os resultados mostram que o tratamento com NAC: 1) diminuiu a latência para achar a plataforma no labirinto aquático (MWM) e aumentou a latência para descer da plataforma na esquiva passiva (SDPA) dos animais que apresentaram-se alteradas nos animais que receberam STZ; 2) restaurou a atividade enzimática da AChE e butirilcolinesterase (BChE) e os níveis de acetilcolina (ACh) corticais e hipocampais potencializadas pela STZ; e 3) protegeu do desequilíbrio do metabolismo energético cerebral induzido pela STZ. Finalmente, foi avaliado o efeito da NAC sobre o déficit cognitivo a longo prazo em camundongos. Para tal, os animais foram tratados com NAC (5 mg/kg/dia v.o.) ou salina por 30 dias consecutivos e com STZ (2.5 mg/kg i.c.v.) ou FCEa no primeiro e terceiro dias. Um tratamento com fisostigmina (PHY; 0,25 mg/kg/dia v.o.) foi realizado em paralelo como medida de controle positivo. Totalizando assim seis grupos. Ambos os tratamentos com NAC e PHY: 1) diminuíram a latência para achar a plataforma no labirinto aquático (MWM) e aumentaram o tempo de exploração no novo objeto (NOR) que apresentaram-se alteradas nos animais que receberam STZ; 2) normalizaram a atividade enzimática da AChE cortical e hipocampal potencializada pela STZ; 3) resgataram a plasticidade sináptica, recuperando os níveis de sinaptofisina (SYN), proteína associada aos microtúbulos do tipo 2 (MAP2) e proteína ácida fibrilar glial (GFAP) diminuídas pela STZ. Desta forma, o tratamento com NAC protegeu do déficit cognitivo induzido pela STZ em camundongos, normalizando a atividade colinérgica e reestabelecendo a plasticidade sináptica.

Palavras chave: cognição; colinesterase; anticolinesterásico; inibidor não-competitivo.

[1] Conjunto de práticas de assistência ao paciente incurável que visa oferecer dignidade e diminuição de sofrimento.

ABSTRACT

N-ACETYLCYSTEINE EFFECT ON THE COGNITIVE DEFICT INDUCED BY STREPTOZOTOCIN IN MICE

AUTHOR: Michael da Costa
ADVISOR: Maria E. Pereira
CO-ADVISOR: Ricardo Brandão
CO-ADVISOR: Cecília M.P. Rodrigues

Cognitive impairment is a mental disorder which is associated with neurodegenerative diseases such as Alzheimer's disease (AD), which is the most common form of dementia. Currently there are no consistent evidence who allow support any measure for the prevention of this disease (DAVIGLUS et al., 2010). As for its treatment, there are methods which can provide relative relief of the symptoms, however, only of palliative nature. Thus, this study aimed to evaluate the n-acetylcysteine (NAC), a molecule with neuroprotective properties, in the treatment and prevention of subsequent dementia of AD, using an experimental model of dementia induced by streptozotocin (STZ) in mice. Initially the effect of NAC on the activity of acetylcholinesterase (AChE) activity in vitro mouse brain was evaluated. The ideal dithiobisnitrobenzoate (DTNB) concentration and pH was 0.3 mM and 7.4, respectively. Linearity in enzyme activity was obtained at acetylthiocholine (ATCh) concentrations ranging from 0.025 to 0.450 mM. Sixty sec prior to the addition of ATCh range to avoid interference DTNB NAC interaction was added to the method. NAC interfered with AChE V_{max} starting at the concentration of 75 uM without affecting the K_m, featuring a non-competitive inhibition. In a second instance, we evaluated the NAC effect on the short-term cognitive impairment in mice. For this, the animals were divided into four groups and were treated with NAC (50 mg/kg /day v.o.) or saline for nine consecutive days, and with STZ (2.5 mg/kg i.c.v.) or aCSF at the first and third days. The results show that NAC treatment: 1) normalized the latency to find the platform in the water maze (MWM) and to get off the platform in passive avoidance (SDPA) that had been altered in animals that received STZ; 2) normalized the AChE and butyrylcholinesterase (BChE) and restored the acetylcholine (ACh) levels in cortical and hippocampal enzymatic activity potentiated by STZ; 3) protected the brain energy metabolism imbalance induced by STZ. Finally, we evaluated the effect of NAC on the long-term cognitive impairment in mice. To this end, animals were treated with NAC (5 mg/kg/day v.o.) or saline for 30 consecutive days, and with STZ (2.5 mg/kg i.c.v.) or aCSF the first and third days. A treatment with physostigmine (PHY; 0.25 mg / kg / day V.O.) was done in parallel as a positive control measure. Totalizing six groups. Both treatments with NAC and PHY: 1) reduced the latency to find the platform in the water maze (MWM) and increased exploratory time on the new object task (NOR) of those animals that received STZ; 2) normalized the cortical and hippocampal AChE enzymatic activity enhanced by STZ; 3) rescued the synaptic plasticity, recovering the synaptophysin (SYN), microtubule-associated protein type 2 (MAP2) and glial fibrillary acidic protein (GFAP) levels reduced by STZ. Thus, the NAC treatment protected from the cognitive impairment induced by STZ in mice, normalizing the cholinergic activity and reestablishing synaptic plasticity.

Key-words: cognition; cholinesterase; anticholinesterase; noncompetitive inhibitor.

LISTA DE ILUSTRAÇÕES

LISTA DE TABELAS

LISTA DE ABREVIATURAS E SIGLAS

ABTS	Ácido 2,2’-azinobis(3-etilbenzenotiazolina- 6-sulfônico)
Acetil-CoA	Acetil coenzima A
ACh	Acetilcolina
AChE	Acetilcolinesterase
ADN	Ácido desoxirribonucleico
ADO	Adenosina
Ala	Alanina
ASCs	Sistema alanina-serina-cisteína
ATCh	Acetiltiocolina
ATP	Adenosina trifosfato
βA	Peptídeo β-amilóide
BCh	Butirilcolina
BChE	Butirilcolinesterase
BHE	Barreira hematoencefálica
BTCh	Butiriltiocolina
BzCh	Benzoilcolina
Carb	Carbamatos
Ch	Colina
ChAT	Colina acetiltransferase
ChEs	Colinesterases
ChOx	Colina oxidase
DA	Doença de Alzheimer
DTNB	Ditiobisnitrobenzoato
ECRI	Estado cerebral resistente à insulina
EO	Estresse oxidativo
E.C.	Classificação enzimática
GFAP	Proteína ácida fibrilar glial
Glu	Glutamato
GLUT2	Transportador de glicose tipo 2
Gly	Glicina
His	Histidina
IBA1	Molécula adaptadora ligante de cálcio ionizado-1
iChEs	Anticolinesterásicos

i.c.v.	Via intracerebroventricular
k	Constante enzimática
K_m	Constante de Michaelis-Menten
kDa	Quilo Dalton
MAP2	Proteína associada aos microtúbulos do tipo 2
MCh	Metilcolina
MnSOD	Superóxido dismutase dependente de manganês
MPO	Mieloperoxidase
NAC	N-acetilcisteína
NAD^+	Dinucleótido de nicotinamida e adenina
NPSH	Tióis não-proteicos
OPs	Organofosforados
PARP	Poli (ADP-ribose) polimerase
PCh	Propionilcolina
PHY	Fisostigmina
pTrkB	Receptores tirosina quinase B fosforilado
S.E.M.	Erro padrão médio
Ser	Serina
SH	Grupamento sulfídrico (Tiol)
SNA	Sistema nervoso autonômico
SNC	Sistema nervoso central
SNP	Sistema nervoso periférico
STZ	Estreptozotocina
SYN	Sinaptofisina
Trp	Triptofano
TSH	Tióis totais
VAChT	Vesículas transportadoras de acetilcolina
V_{max}	Velocidade máxima

LISTA DE APÊNDICES

SUMÁRIO

1 INTRODUÇÃO

1.1 Memória

A memória é o processo na qual a informação é codificada, armazenada e recuperada. A codificação permite que as informações externas, em forma de estímulos químicos e físicos, cheguem através dos cinco sentidos. Já o armazenamento permite a retenção desta informação por um período de tempo. E a recuperação permite que esta informação seja localizada e volte à consciência. A memória pode ser classificada em três ramos principais: 1) Memória sensorial: permite armazenar informação sensorial por um período menor ao de um segundo após a sua percepção, fugindo assim do controle cognitivo e assim sendo considerada como como uma resposta automática (SPERLING, 1963). 2) Memória de curto prazo: permite a recuperação da informação após um período de alguns segundos a poucos minutos. 3) A memória de longo prazo: permite armazenar e evocar uma maior quantidade de informação por um período maior de tempo em relação à memória de curto prazo (MILLER e GEORGE, 1956).

Enquanto a memória de curto prazo é suportada pelas regiões neuronais dependentes do lobo frontal do córtex cerebral, como por exemplo o dorsolateral, o pré-frontal e o parietal, a memória de longo prazo por outro lado é suportada pelas conexões neuronais de todo o cérebro, em especial do hipocampo (SCOVILLE e MILNER, 1957). Dentro da memória de longo prazo encontra-se a memória explicita ou declarativa que faz referência a informações que podem ser conscientemente recuperadas[2] e a memória implícita ou procedural, a qual faz referência a informações inconscientes[3]. A memória juntamente de uma série de habilidades mentais relativas ao conhecimento faz parte do processo de cognição.

1.2 Cognição

A cognição é a ação mental ou processo de aquisição de conhecimento e compreensão através da experiência e os sentidos. Esta engloba processos como conhecimento, atenção, compreensão, raciocínio, julgamento, avaliação e memória. Os processos cognitivos podem ser naturais ou artificiais, assim como, consciente ou inconsciente. Desta forma pode ser analisado por diferentes perspectivas e contextos, na neurologia, psicologia, filosofia e ciências computacionais.

[2] Fatos ou eventos como por exemplo: aniversários ou eventos esportivos.
[3] Habilidades para ações como por exemplo: amarrar os cadarços ou andar de bicicleta.

1.2.1 Distúrbio cognitivo

O distúrbio cognitivo, também chamado de déficit cognitivo ou comprometimento cognitivo é um termo abrangente para descrever qualquer característica que aja como uma barreira para o processo de cognição (COREN e col., 1999). Algumas das principais causas do déficit cognitivo em seres humanos são as doenças neurodegenerativas, como a doença de Alzheimer (DA) que compreende 50 a 70% de todos os casos (FERRI e col., 2005). Esta é caracterizada por um comportamento de progressiva perda da memória, pensamento, compreensão, cálculo, linguagem, capacidade de aprendizagem e julgamento (GIULIANIA e col., 2009).

A DA foi descoberta em 1907 pelo Dr. Alois Alzheimer, o qual a descreveu como uma doença neurológica progressiva, neurodegenerativa e irreversível. Diferentemente do que acontece no envelhecimento, na DA o progressivo declínio intelectual é muito mais acentuado (STIX, 2010). Durante os estágios iniciais ocorre uma perda indiciosa da memória para fatos recentes, podendo também ocorrerem efeitos secundários, como depressão, mudança de personalidade e ansiedade. Já disfunções motoras, dificuldades na linguagem e deficiência sensorial são observados em estágios mais avançados da doença (DOOLEY e LAMB, 2000; STIX, 2010).

No que arremete as suas características clínicas, a DA está associada com mudanças neuropatológicas, como a formação de placas senis, consequência do depósito de fragmentos β-amilóide (βA) (HARDY e SELKOE, 2002) e com a morte progressiva de neurônios, em particular, no córtex cerebral e hipocampo (XU e col., 2006). Este processo neurodegenerativo é acoplado à disfunção mitocondrial, metabolismo energético prejudicado e ativação de vias de sinalização pró-apoptóticas (MANCUSO e col., 2009). Existindo também evidências de que a produção aumentada de radicais livres[4] possam desempenhar um papel importante no desenvolvimento da doença (MARKESBERY e col., 1997).

Acredita-se que, no início da patogênese da DA, o controle do metabolismo neuronal da glicose seja perturbado pela insuficiência na transdução de sinal desencadeada pela insulina (FRÖLICH e col., 1998), a qual controla os processos de liberação de neurotransmissores nas sinapses e ativa as vias de sinalização associadas com a aprendizagem e a memória de longo prazo (KLEIN, 2002). Desta forma, alterações graves no metabolismo da glicose são comuns nas formas de demência esporádica[5] decorrentes da DA. Esta alteração no metabolismo energético pode originar

[4] Átomo ou molécula que contém um elétron (*e*-) desemparelhado na sua última camada orbital. Exemplo: radical hidroxila ($HO^{\bullet}$), radical peróxido ($O_2^{\bullet -2}$) e radical ânion superóxido ($O_2^{\bullet -}$) entre outros.

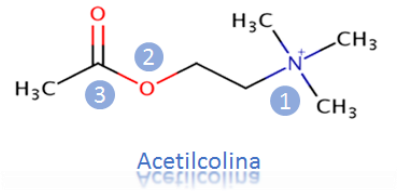
[5] Na qual o fator hereditário não é óbvio. Difere da forma familiar na qual a doença é transmitida através das gerações.

disfunções cognitivas por reduzir a síntese de adenosina trifosfato (ATP) e acetil-coenzima A (acetil-CoA), levando assim a uma redução na atividade da colina acetiltransferase (ChAT). Isto juntamente com o aumento na atividade das cholinesterases (ChEs) no sistema nervoso central (SNC) (SHARMA e col., 2008; SHOHAM e col., 2007) leva a diminuição da concentração de acetilcolina (ACh).

1.3 Acetilcolina (ACh)

A ACh é um éster do ácido acético e da colina (Figura 1) com um peso molecular de 146,2 g/mol o qual foi descoberto por Henry Hallet Dale em 1914, quem estudou seus efeitos sobre o tecido cardíaco (RANG e col., 2007). A ACh é principalmente um neurotransmissor do sistema nervoso autonômico (SNA). Contudo, também age no sistema nervoso periférico (SNP) e SNC (LI e col., 2014). No SNP a ACh estimula o músculo esquelético ao ligar-se aos receptores nicotínicos presentes nas fibras musculares, abrindo assim os canais de sódio (Na) na membrana celular e produzindo a contração muscular. Já no SNC ela está principalmente envolvida na aprendizagem e na memória.

Figura 1. Estrutura da ACh {[2-(acetiloxi)etil]trimetilamônio}.



Demonstrando grupamentos: amônia quaternária (**1**), éster (**2**) e colina (**3**)[6].

A ACh é sintetizada a partir da acetil-CoA e da colina (Ch) em um processo catalisado pela ChAT nos terminais axonais (Figura 2). Em mamíferos a acetil-CoA utilizada na síntese da ACh provem principalmente da oxidação do piruvato[7]. Estima-se que aproximadamente a metade da Ch utilizada na síntese da ACh provenha da reciclagem desta após ser hidrolisado na fenda sináptica. Uma segunda fonte de Ch vem da quebra da fosfatidilcolina extracelular. Estas duas vias de receptação de Ch são fundamentais para o SNC, uma vez que a Ch presente na corrente sanguínea não pode cruzar a barreira hematoencefálica (BHE) (SIEGEL e col., 1999). Desta forma, as

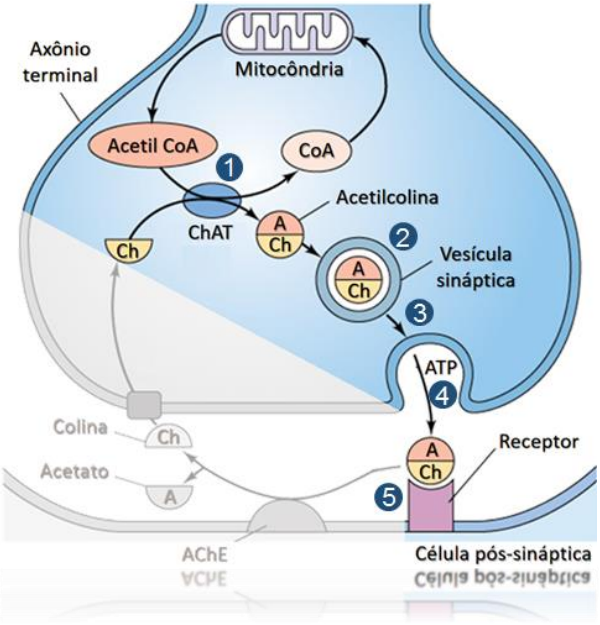
[6] Fonte: DrugBank (http://www.drugbank.ca/drugs/DB03128).
[7] Produto final da oxidação da glicose durante a glicólise aeróbica.

concentrações de ACh no SNC são sujeitas a variações, enquanto que nos músculos são mais constantes.

Após a síntese da ACh, esta é armazenada e transportada pelos transportadores denominados VAChT[8] em vesículas até a terminação do nervo (PARSONS e col., 1987). A captação do neurotransmissor pela vesícula é mediada por uma bomba de prótons. Já para a liberação de ACh se requer a presença extracelular de cálcio (Ca^{2+}) o qual entra ao neurónio durante sua despolarização. Uma vez liberada ao meio, a ACh pode ligar-se tanto aos receptores nicotínicos[9] como muscarínicos[10].

É importante ressaltar que nem toda ACh sintetizada é liberada ao meio. Estudos mostram que existem dois ¨pools¨ de ACh na célula, estando somente um destes disponível para liberação denominado depósito[11]. O outro denominado estacionário[12], servirá como reserva e irá recarregando o já liberado (SIEGEL e col., 1999).

Figura 2. Síntese e liberação da ACh.



A síntese da ACh a partir da Ch e acetil-CoA é catalisada pela ChAT (**1**), logo o neurotransmissor é armazenado nas vesículas (**2**) e transportado à membrana para serem liberadas (**3**) juntamente com o ATP (**4**), uma vez no espaço sináptico a ACh irá ligar-se ao receptor na célula pós-sináptica (**5**).

Após sua ligação ao receptor e transmissão do impulso nervoso, a ACh é hidrolisada. Contudo, a hidrólise desta pode promover a agregação de βA (LINSTON e

[8] VAChT: transportador vesicular de acetilcolina (do inglês: ¨*vesicular acetylcholine transporter*¨).
[9] Receptor ionotrópico que permitem a entrada de íons à célula após a ligação ao neurotransmissor (placa motora).
[10] Receptor metabotróficos o qual gera mensageiros secundários após a ligação ao neurotransmissor (músculo liso).
[11] Do inglês "*depot*".
[12] Do inglês "*stationary*".

col., 2004). Além disso, os níveis de ACh estão altamente associados com a disfunção colinérgica presente na DA e também observada na doença de Parkinson (AULD e col., 2002).

1.4 Colina acetiltransferase (ChAT)

A colina acetiltransferase ou acetilcolinatransferase [(ChAT), E.C.: 2.3.1.6][13] é uma enzima pertencente à família das transferases[14] e catalisa a transferência reversível do grupo acetil entre a acetil-CoA e a Ch formando a ACh.

Esta enzima não é saturada aos níveis de substrato encontrados nas terminações neuronais, podendo trabalhar no seu máximo cinético. Desta forma, sendo a disponibilidade de substratos um fator limitante para a síntese do neurotransmissor (TUĈEK, 1990).

A ChAT é sintetizada no pericário[15] dos neurônios colinérgicos e transportadas para as terminações neuronais. Em primatas[16] existem duas variantes desta enzima, uma com peso de 69 kDa e outra com peso de 82 kDa (ODA, 1999; DOBRANSKY e RYLETT, 2003). Ainda não se sabe se estas transcrições desempenham funções fisiológicas diferentes ou se regulam diferentes mecanismos celulares. O que já foi demonstrado é que a transcrição 82 kDa localiza-se no núcleo celular diferentemente da 69 kDa que é encontrada no citoplasma (DOBRANSKY e RYLETT, 2003).

Diferentes mutações da ChAT têm sido isoladas de diferentes espécies e toda mutação resulta em perda de atividade catalítica, sendo até de 98% em alguns casos. As mutações desta enzima têm sido altamente associadas com doenças neurodegenerativas como Huntington, esclerose amiotrófica lateral e DA (CAI e col., 2004).

1.5 Colinesterases (ChEs)

As ChEs são hidrolases carboxílicas especializadas na hidrolise de ésteres de colina (Ch). Dentro desta classe enzimática são duas as que se destacam; a acetilcolinesterase [(AChE), E.C.: 3.1.1.7][17] e a butirilcolinesterase [(BChE), E.C.: 3.1.1.8][18]. Ambas AChE e BChE incluem-se na subclasse das β-esterases, enzimas as

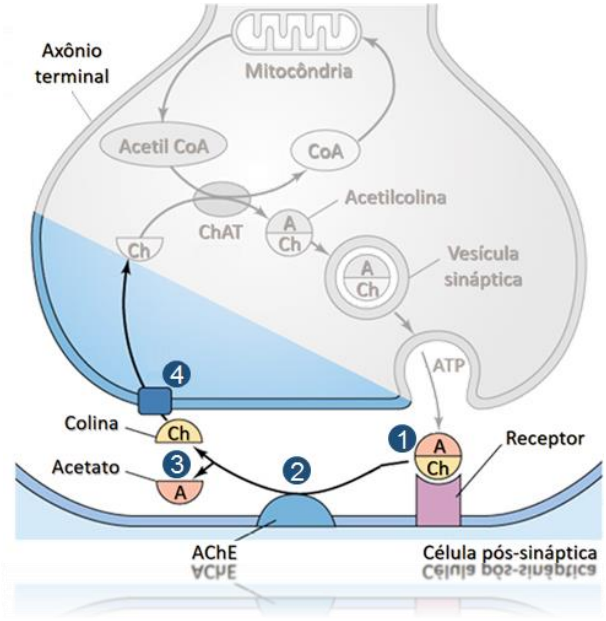
[13] Classificação enzimática: 2 - transferases, 3 - de grupos acil, 1 - de grupos amino, 6 - de colina.
[14] Enzimas que transferem grupos funcionais de uma molécula à outra, ($XA + Y \rightarrow X + YA$).
[15] Centro trófico do neurônio, onde encontram-se o núcleo e demais organelas.
[16] Ordem que compreende os mamíferos popularmente chamados de macacos, símios, lêmures e os seres humanos.
[17] Classificação enzimática: 3 - hidrolases, 1 - de ligações ésteres, 1 - de ésteres carboxílicos, 7 - de acetila.
[18] Classificação enzimática: 3 - hidrolases, 1 - de ligações ésteres, 1 - de ésteres carboxílicos, 8 - de butirila.

quais podem ser inibidas por compostos das classes dos organofosforados[19] (OPs) e carbamatos[20] (Carb) (ALDRIDGE e REINER, 1972).

A outra subclasse é denominada α-esterases na qual estão incluídas as arilesterases, paraxonases e DFPases, e estas são capazes de hidrolisar compostos como os OPs e Carb. Representando assim, uma importante linha de detoxificação (FURLON e col., 2000; LA DU e col., 1999). A distribuição, assim como as funções fisiológicas daquelas duas β-esterases nos diferentes organismos são bem diferentes (ECOBICHON, 1996).

A AChE é principalmente encontrada no SNC, junções neuromusculares e no sistema hematopoiético em vertebrados, possuindo uma função fundamental na neurotransmissão colinérgica. A sua principal função é hidrolisar o neurotransmissor ACh após o impulso sináptico ter sido formado, evitando assim, uma excessiva estimulação dos neurónio pré e pós-sinápticos (ANDERSON e KEY, 1999) (Figura 3).

Figura 3. Hidrólise da ACh pela AChE no espaço sináptico.



Após ligar-se ao receptor (**1**) e transmitir o impulso nervoso, a ACh é hidrolisada pela AChE (**2**) formando como produtos o acetato e a Ch (**3**) esta última é recaptada pelo transportador de Ch (tCh) (**4**) para a síntese de ACh na célula pré-sináptica[21].

Esta enzima também é encontrada em altas concentrações nas hemácias, porém, sua concentração depende de acordo com a espécie animal. A BChE é

[19] Compostos orgânicos derivados do ácido fosfórico e que contém ligações carbono-fósforo (C-F).
[20] Compostos orgânicos, também chamados de uretanos (NHCOO-), são ésteres do ácido carbâmico (NH_2COOH).
[21] Figura adaptada de CNSforum (https://www.cnsforum.com/educationalresources/imagebank/dementia_cholinergic).

principalmente encontrada no fígado, no plasma sanguíneo, intestinos e rins. Em baixas concentrações esta enzima juntamente com a AChE, também é encontrada nos espaços sinápticos, terminações motoras e fibras musculares (SILVER, 1974).

Acredita-se que a BChE encontrada no plasma sanguíneo esteja envolvida na hidrólise de ésteres ingeridos na dieta, já que algumas plantas dos gêneros das solanáceas[22] e das fabáceas[23], desta última destaca-se o feijão de Calabar[24], possuem glicoalcalóides e esteroides de ocorrência natural os quais produzem efeitos tóxicos ao organismo (KRASOWSKI e col., 1997; McGEHEE e col., 2000).

Contudo, Adler e col. (2004) demonstrou que camundongos *knock-out* para o gene da AChE e com atividade normal da BChE conseguiram sobreviver por mais de um ano. Em estudos similares, Li e col. (2000) e Xie e col. (2000) também obtiveram os mesmos resultados, sugerindo assim que a atividade da BChE pode substituir em parte a atividade da AChE.

O substrato específico da AChE é a ACh. Já a BChE não possui substrato específico, podendo hidrolisar a butirilcolina (BCh), assim como, a propionilcolina (PCh) e a ACh, dependendo da espécie animal (SILVER, 1974). Um exemplo ocorre nos roedores como o rato e também em coelhos e vacas nos quais a atividade da BChE plasmática possui uma maior afinidade para a PCh do que para a BCh.

As ChEs são sintetizadas como monômeros catalíticos globulares (G_1) os quais se oligomerizam via ligações bissulfetos formando as isoformas G_2 e G_4 no reticulo endoplasmático granuloso. Aproximadamente 80% das enzimas são degradadas pelas proteases intracelulares após a translocação do ribossomo ao aparelho de Golgi onde as unidades G_1 são glicosiladas. As formas assimétricas A_4, A_8 e A_{12} são formadas após a ligação de uma cauda de colágeno. Ambas as formas podem ser secretadas ao meio.

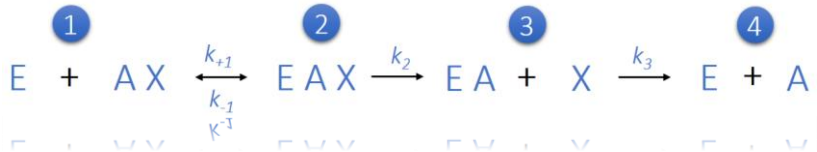
A AChE possui no seu sitio ativo um desfiladeiro[25] com 14 resíduos aromáticos, com a tríade de resíduos catalíticos de glutamato (Glu334), histidina (His447) e serina (Ser203). Já o seu sítio aniônico apresenta resíduos de triptofano (Trp286) e tirosinas 72 e 124. Os elétrons (e^-) presentes neste sitio interagem com o nitrogênio quaternário (N^+) presente na molécula de ACh. Acredita-se que a sequência de aminoácidos no sitio aniônico seja importante para determinar a especificidade de substrato (DOCTOR e col., 1998).

[22] Família botânica de plantas angiospérmicas das quais destacam-se as batatas, tomates e fumo.
[23] Família botânica também conhecida como leguminosa das quais fazem parte os feijões, soja, ervilha e alfafa.
[24] Também chamado de *Physostigma venenosum,* contém o alcaloide inibidor reversível da ChEs, a Fisostigmina.
[25] Do inglês ¨*gorge*¨.

Estruturalmente a AChE e a BChE possuem algumas semelhanças. O sítio ativo da BChE também possui um grande desfiladeiro com muitos resíduos aromáticos e com uma tríade catalítica constituída por resíduos de serina (Ser198), glutamato (Glu438) e histidina (His438) e uma cavidade oxiânion[26] constituídas por glicina (Gly116), glicina (Gly117) e alanina (Ala199) essencial para a função catalítica.

Desta forma, tendo em conta suas similaridades estruturais, é lógico acreditar que o mecanismo catalítico para a hidrólise de ACh da BChE seja o mesmo da AChE. Porém, o passo limitante[27] da reação catalítica da BChE é a acetilação[28], enquanto que para a reação catalítica da AChE é a desacetilação[29] (CHEN e col., 2011) (Figura 4).

Figura 4. Esquema da reação cinética das ChEs.

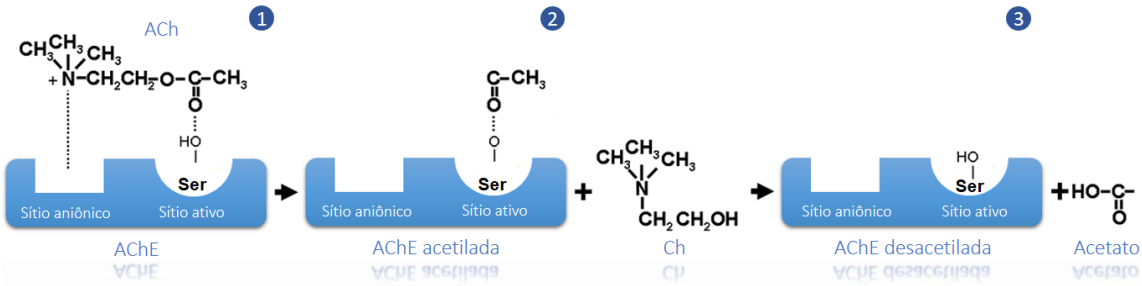


Ligação da enzima com o substrato (**1**) e formação do complexo reversível enzima-substrato (**2**) onde ocorre o processo de acetilação da enzima, etapa limitante da atividade catalítica da BChE (representado pela constante de associação k_2) e formação de subproduto (**3**), seguida do processo de hidrólise onde ocorre a desacetilação, etapa limitante da atividade catalítica da AChE (representado pela constante de dissociação k_3) e formação de produto (**4**). Abreviações - E: enzima (ChE), AX: substrato (ACh ou BCh), EAX: complexo enzima-substrato, EA: enzima acetilada, X: Ch, A: acetato ou butirato[30].

O mecanismo de catálise de ACh pela AChE inicia com o ataque nucleofílico[31] do carbono (C) do grupamento colina, resultando assim, na formação do complexo reversível enzima substrato seguida da acetilação do sítio ativo. Este processo é seguido de uma rápida hidrólise da enzima acetilada produzindo acetato e Ch (Figura 5). Uma unidade da AChE é capaz de hidrolisar 6 x 10^5 moléculas de ACh por minuto, com um tempo de ¨turnover¨[32] de 150 microssegundos (TAYLO, 1996).

[26] Estrutura enzimática que ajuda a estabilizar um oxigênio desprotonado, aumentando a afinidade.
[27] Do inglês ¨*rate-determining stage*¨.
[28] Adição de um grupamento acetila (CH_3CO) a um composto orgânico.
[29] Remoção de um grupamento acetila (CH_3CO) de composto orgânico.
[30] Figura adaptada de ROSEMBERRY e col. (1998).
[31] Reação na qual um nucleófilo (rico em e^-) substitui um elétrofila (pobre em e^-).
[32] Máximo de moléculas de substrato que uma enzima pode converter em produto por sitio catalítico por minuto.

Figura 5. Mecanismo de hidrólise da ACh pela AChE.



Ataque nucleofílico do sitio ativo ao grupamento Ch e atração eletroestática do sitio aniônico ao grupamento N^+ (**1**), seguida da acetilação da AChE (**2**) e rápida desacetilação (hidrólise) da AChE, produzindo acetato e Ch (**3**).

Desta forma, devido principalmente a suas funções no SNC, esta família de enzimas está altamente envolvida nos processos de cognição, aprendizagem e memória, assim como também no que diz respeito a doenças neurodegenerativas as quais venham a prejudicar estes processos (DARVESH e col., 2003; GRON e col., 2006; HUT e VAN DER ZEE, 2011).

Assim, com base na hipótese colinérgica da DA, a qual atribui o declínio cognitivo à perda de neurotransmissão colinérgica no córtex cerebral e hipocampo, o principal foco do desenvolvimento de medicamentos para contrapor a DA nas últimas décadas tem sido sobre as estratégias que melhorem a função colinérgica. Entre elas está: 1) a terapia de reposição de ACh com precursores desta, como a colina e a lecitina; 2) o uso de agonistas muscarínicos como a xanomelina e sabcomelina, e de agonistas nicotínicos como a nicotina; 3) através do aumento da produção de ACh utilizando-se de fosfatidilserina e 4) utilização de inibidores das ChEs (iChEs). Contudo, esta última parece ser a única terapia que tem demonstrado resultados positivos consistentes no tratamento da DA (HOLDEN e KELLY, 2002).

1.6 Anticolinesterásicos (iChEs)

Os iChEs são compostos de origem natural ou artificial que possuem a capacidade de inibir a atividade das ChEs, aumentando as concentrações de seus substratos no meio. A simples inibição da enzima não tem por si um efeito farmacológico. Contudo, o efeito farmacológico dos iChEs se deve ao aumento da concentração de ACh e ao tempo de ação maior desta nos receptores.

Os iChEs ganharam sua atenção por parte dos investigadores na década de 70, em modelos experimentais de indução de amnésia (BARTUS, 1978). Com a descoberta da depleção da ACh no hipocampo (SMITH e SWASH, 1978) e dos efeitos benéficos

sobre a cognição (PETERS e LAVIN, 1979; MURAMOTO e col., 1979) por parte da fisostigmina (PHY), o uso de diferentes iChEs como potenciais agentes terapêuticos contra a DA foi posto em prática em testes experimentais. Hoje em dia, inúmeros são os iChEs utilizados no tratamento de doenças neurodegenerativas, assim como outras enfermidades, como miastenia e glaucoma. Alguns destes compostos são descritos a continuação.

A PHY citada acima, foi o primeiro composto desta classe da qual se teve conhecimento dos seus usos farmacológicos (MARQUARDT e col., 1999). Hoje em dia a PHY pode ser obtida a partir da triptamina[33]. Por ser uma amina terciária, esta pode atravessar a BHE e hoje é utilizada no tratamento do glaucoma[34], gastroparesia[35], hipotensão ortostática[36] e DA. Os efeitos colaterais desta incluem a depressão.

A tacrina foi a primeira droga dessa classe a ser experimentada com êxito em seres humanos. Apesar de um início muito promissor seu uso foi descontinuado devido a sua toxicidade ao tecido hepático (WATKINS e col., 1994). Além disso, a sua toxicidade dependente da dose foi responsável por vários efeitos colinérgicos colaterais, como náusea, vômito, diarreia e dor abdominal.

O donepezil foi a segunda medicação aprovada, nos Estados Unidos, para o tratamento da DA. O estudo que serviu de base para a introdução da droga no mercado americano foi publicado por Roger e col. (1998). O donepezil possui uma alta afinidade pela AChE e uma afinidade muito baixa pela BChE apresentando eficácia no tratamento da DA em doses de 5 a 10 mg. Alguns efeitos adversos são a fadiga, náuseas, tontura, diarreia e renite. Contudo, apesar do donepezil parecer ser bem tolerado pela maioria dos pacientes seu uso foi ocasionalmente descontinuado no tratamento da DA (ALMEIDA, 1998).

A rivastigmina é outro iChEs utilizado no tratamento da DA. Esta é considerada um inibidor pseudo-irreversível, pois sua interação com a enzima leva a formação de um produto de rápida excreção, e um outro de longa duração, porém reversível, o qual impede a hidrólise da ACh. Desse modo o efeito dessa droga perdura mesmo após a eliminação da droga-mãe do organismo, reduzindo os riscos de interação com outros medicamentos (IBACH e HAEN, 2004). A dose recomendada desta droga é de 12 mg, divididas em duas ou três doses diárias. Contudo, náusea, vômito, diarreia, sincope[37],

[33] Alcaloide (C-N-O-H) monoamínico bioativo encontrado em plantas, fungos e animais.
[34] Grupo de doenças que envolvem pressão intraocular associada a neuropatia óptica.
[35] Paralisia parcial do estomago, caracterizada pela lentidão na passagem de alimentos neste.
[36] Redução excessiva da pressão arterial ao adoptar-se a posição vertical.
[37] Perda súbita da consciência e consequentemente da postura.

dores abdominais e tonturas em pacientes com longos períodos de tratamento ou que ingeriram altas doses de rivastigmina foram relatadas.

O metrifonato é um iChEs irreversível. A eficácia desta droga no tratamento de pacientes no tratamento da DA foi descrita por Becker e col. (1998). A dose diária ideal desta era de 2 mg/kg nos primeiros cinco dias e logo de 3 mg/kg semanais. Alcançando-se 60% de inibição da atividade da AChE eritrocitária a partir do sétimo dia. Morris e col. (1997) revelou que, além dos efeitos benéficos sobre a cognição, o metrifonato também contribuiu para melhorar o comportamento dos pacientes. Contudo, o metrifonato tem como princípio ativo o 2,2,2-tricloro-1-hidro-xietil dimetil sulfonato, o qual é utilizado como inseticida em vários tipos de culturas e pode causar neuropatia retardada. Já seus principais efeitos colaterais se remetem a diarreia, dores abdominais e náuseas (MORRIS, 1998).

Finalmente a galantamina, um alcaloide originalmente extraído dos bulbos da anêmona caucasiana e do narciso *Galanthus niralus*[38]. Esta é considerada um iChEs de ação rápida. Há aproximadamente 40 anos vem sendo usada no tratamento de miastenia grave[39] e na reversão farmacológica do bloqueio neuromuscular. A dose diária pode variar de 4 a 24 mg/kg e sua biodisponibilidade é de 80 a 100%, possuindo um período de vida média de 7 horas. A estimulação dos receptores nicotínicos também foi proposta como mecanismo de ação adicional, sendo considerado um efeito relevante para o tratamento da DA (DAIAS-BAILADOR e col., 2003). Os efeitos adversos da galantamina são similares aos de outros iChEs, no sentido de que tendem a produzir efeitos gastrointestinais agudos.

1.7 N-acetilcisteína (NAC)

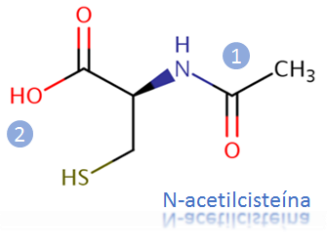
A NAC (Figura 6) é uma molécula derivada do aminoácido cisteína, clinicamente utilizada no tratamento de doenças respiratórias, envenenamento por paracetamol e prevenção de nefropatias (SANSONE e SANSONE, 2011; BERK e col., 2013). Estas aplicações da NAC estão relacionadas a sua habilidade de suprir as defesas antioxidantes do organismo durante situações de estresse, infecções, intoxicações e processos inflamatórios (DEKHUJIZEN, 2004).

O produto do metabolismo da NAC é transportado principalmente pelo sistema alanina-serina-cisteína (ASCs) na maioria das células (ISHIGE e col., 2005). Contudo, a NAC é uma molécula muito permeável às membranas celulares, não necessitando de

[38] Planta herbácea natural da Europa e Ásia, pertencente à família das *Amaryllidaceae*.
[39] Enfermidade neuromuscular crônica autoimune que provoca debilidade dos músculos esqueléticos.

transporte ativo[40] ou co-transporte[41] (SEN, 1997). Uma vez dentro da célula, a NAC sofre hidrolise liberando a cisteína, a qual serve de substrato para a síntese de glutationa (MEISTER, 1995).

Figura 6. Estrutura da NAC {(2R)-2-acetamido-3-ácido sulfanilpropanoico}.



Demonstrando grupamentos: colina (**1**) e sulfídrico (**2**)[42].

Após 2 horas, a NAC administrada por via oral atinge sua maior concentração nos rins, fígado, pulmões, baço, sangue, músculo estriado e cérebro em ratos (SHEFFNER e col., 1996). Contudo, ainda há controvérsias enquanto a sua habilidade em cruzar a BHE, uma vez que, diferentes rotas e doses poderiam resultar em diferentes concentrações viáveis. A ^{14}NAC quando administrada por via intra-arterial (carótida) ou intravenosa (jugular) consegue atravessar a BHE em uma grande porcentagem (McLELLAN e col., 1995). Já elevados níveis de NAC foram mensurados no cérebro após a administração deste composto por via oral e intraperitoneal em roedores (SAMUNI e col., 2013).

A NAC possui uma vida média de aproximadamente 6 horas (SANSONE e SANSONE, 2011). Além de agir como precursora da glutationa no sistema antioxidante, seus efeitos anti-inflamatórios, anti-apoptóticos e pró-neurogênicos têm sido também relatados (ASEVEDO e col., 2014). Desta forma, o uso da NAC como agente neuroprotetor foi proposto, uma vez que estas vias estão implicadas na neuroprogressão (DOOD e col., 2013). Um resumo dos mecanismos de ação pelo qual a NAC age em distúrbios neurodegenerativos pode ser observado na tabela 1.

[40] Transporte ativo de uma molécula de região de baixa concentração para uma região de alta concentração realizada por intermédio de dispêndio de energia.
[41] Transporte contra um gradiente eletroquímico de uma molécula, por intermédio de dispêndio de energia fornecida pelo transporte a favor de gradiente de uma segunda molécula.
[42] Fonte DrugBank (http://www.drugbank.ca/drugs/DB06151).

Tabela 1. Mecanismos de ação da NAC em diferentes desordens neurodegenerativas[43].

Doença	Mecanismo
Doenças neurodegenerativas	Atividade antioxidante e aumento dos níveis de glutationa.
Síndrome de Down	Aumento e modulação dos níveis de superóxido.
Esclerose múltipla	Diminuição de radicais livres e inibição de TNF.
Esclerose amiotrófica	Aumento dos níveis de glutationa peroxidase e diminuição dos níveis de radicais livres.
Doença de Parkinson	Aumento dos níveis de glutationa e diminuição dos níveis de radicais livres.
Doença de Huntington	Diminuição dos níveis de radicais livres e prevenção da disfunção mitocondrial.
Doença de Alzheimer	Aumento dos níveis de glutationa.
Isquemia cerebral focal	Inibição da NOS, regeneração do fator relaxante endotelial, aumento dos níveis de glutationa, aumento da microcirculação sanguínea.
Hemorragia subaracnóidea	Diminuição dos níveis de radicais livres, inibição apoptótica, aumento dos níveis de glutationa e a atividade da superóxido dismutase.
Traumatismo cerebral	Aumento dos níveis de glutationa, inibição da ativação da NF-*k*B e TNF-α.

Assim, o uso da NAC em tratamentos relacionados à cognição já vem sendo estudado nos últimos anos. Recentemente seu efeito sobre déficit de memória espacial induzido pelo ácido glutárico e lipolpolissacarídeos foi relatado por Rodrigues e col. (2013). Em combinação com a minociclina a NAC melhorou os aspectos comportamentais de ratos em um modelo leve de traumatismo cranioencefálico (HABER e col., 2013). Já Thakurta e col. (2014) mostraram os efeitos positivos da administração da NAC em combinação com outros antioxidantes como o ácido α-lipóico e α-tocoferol no declínio cognitivo consequente do envelhecimento em ratos. No entanto, escassos são os estudos onde são demonstrados os efeitos da NAC em modelos de déficit cognitivo induzido por estreptozotocina (STZ).

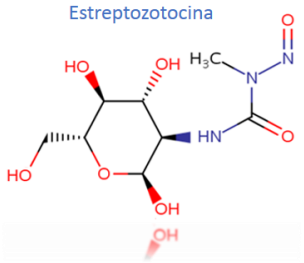
1.8 Estreptozotocina (STZ)

A STZ é uma nitrosamina[44] (Figura 7) de ocorrência natural que é particularmente tóxica às células betas pancreáticas em mamíferos. Foi originalmente identificada em 1959 como um antibiótico (VAVRA e col., 1959), descoberta a partir do *Strptomyces achromogenes*[45] encontrado na soja.

[43] Tabela adaptada de SHAHRIPOUR e col. (2013).
[44] Composto cancerígeno de fórmula química R_2N-N=O, encontrados em alimentos como cerveja, carnes e queijos.
[45] Bactéria gram-positiva pertencente às Actionobactérias às quais possuem DNA rico em guanina e citosina.

Esta tem sido largamente utilizada como indutora de diabetes melito em animais experimentais, já que esta é captada pelos transportadores de glicose (GLUT 2) presentes nas células pancreáticas. Uma vez dentro da célula a STZ aquilata[46] o DNA, o que leva à morte celular consequência da ativação da poli (ADP-ribose) polimerase (PARP) e da depleção da NAD^+ e dos depósitos de ATP. Contudo, também foi utilizada no tratamento de câncer ao nível das Ilhotas de Langherans, em casos 1nos quais o tumor não pôde ser removido cirurgicamente (MURRAY-LYON e col., 1968). A dose média diária de STZ para este tipo tratamento é de 0,5 mg por via intravenosa, por 5 dias contínuos, repetindo-se a cada 4 a 6 semanas.

Figura 7. Estrutura da STZ {3-metil-3-nitroso-1-[(2S,3R,4R,5S,6R)-2,4,5-triidroxi-6-(hidroximetil)oxan-3-il]urea}[47].



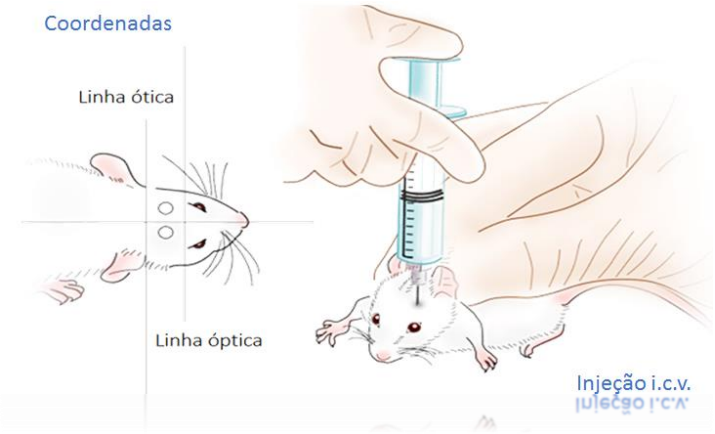
Por outro lado, a injeção intracerebroventricular (i.c.v.) de STZ (Figura 8) em uma dose subdiabetogênica tem sido relatada como um modelo de DA. O pioneiro deste modelo foi o professor Sigfried Hoyer, quem inicialmente observou que na DA existia uma queda no consumo de oxigênio e glicose cerebrais (HOYER e col., 1994). E desta forma acreditou que a maior perturbação bioquímica na DA concernia ao controle metabólico da glicose cerebral, a qual procederia a uma falha de transdução de sinal dos receptores de insulina. Assim, em uma segunda instância, investigou se a utilização local da glicose cerebral após a administração i.c.v. de STZ (1,5 mg/kg) em ratos era afetada (DUELLI e col., 1994).

O seu, juntamente com outros experimentos, mostraram que a aplicação i.c.v. de STZ levava a uma diminuição da utilização de glicose no cérebro, principalmente no córtex frontal e parietal, na concentração de ATP e fosfocreatina, e na razão ATP/ADP (NITSCH e HOYER, 1991; LANNER e HOYER, 1998). Produzindo assim uma série de distúrbios metabólicos (GRIEB, 2016), como a disfunção colinérgica, caracterizada pela inibição da atividade da ChAT e pelo aumento da atividade da AChE no SNC e SNP (SHARMA e col., 2008; SHOHAM e col., 2007) e distúrbios comportamentais, como as

[46] Transferência de um grupamento alquila de uma molécula a outra. Este grupamento pode ser transferido como radicais livres.
[47] Figura extraída de DrugBank (http://www.drugbank.ca/).

disfunções cognitivas (SHARMA e col., 2010). Assim é considerado como um modelo pré-clínico não-transgênico da DA, no qual há um estado cerebral resistente à insulina (ECRI) (GRIEB, 2016).

Figura 8. Injeção de STZ aplicada a ¨mão livre¨ pela via i.c.v.[48]



Uma das principais hipóteses para o desencadeamento destes distúrbios metabólicos e comportamentais provocados pela STZ, é a de que quando injetada por via i.c.v., a STZ dessensibiliza ou danifica os receptores de insulina do cérebro. Ou de outra forma, produz resistência à insulina no cérebro devido a uma resposta inadequada dos receptores. Contudo, esta é somente uma analogia entre o mecanismo de ação da STZ via i.c.v. e o mecanismo de ação da STZ via periférica (SALKOVIC-PETRISIC e col., 2013). Além do mais, devido as diferenças entre os efeitos da STZ quando administrada por vias diferentes, o conceito de dessensibilização ou dano permanente dos receptores de insulina causada pela difusão da STZ pelo parênquima cerebral não parece ser plausível (GRIEB, 2016).

Assim, apesar do fato da administração i.c.v. de STZ produzir distúrbios metabólicos, neuropatológicos e comportamentais em roedores, similares aos encontrados na DA em humanos, ainda se desconhece um mecanismo que possa vir a explicar este fenômeno. Apesar disto, hoje em dia este modelo animal é um dos mais utilizados para o estudo tanto das causas possíveis da doença como para a busca de novas terapias para o tratamento desta.

[48] Figura adaptada de http://www.clodrosome.com/routes-of-administration/intracerebral-injection/

2 OBJETIVO

2.1 Objetivo geral

Avaliar o efeito neuroprotetor da NAC na terapia do déficit cognitivo em um modelo químico de DA induzido pela STZ em camundongos, através de análises comportamentais e bioquímicas.

2.2 Objetivos específicos

- ✓ Padronizar o método de Ellman e col. para avaliar o efeito da NAC (composto contendo -SH) sobre a atividade da AChE cerebral de camundongos in vitro;

- ✓ Avaliar a interferência da NAC sobre o efeito da STZ na cognição e nos parâmetros colinérgicos;

- ✓ Investigar se a NAC protege da neurodegeneração induzida pela STZ.

3 RESULTADOS

Os resultados referentes a esta tese estão apresentados na forma de três protocolos científicos. Estes foram aceitos (artigo) ou estão submetidos (manuscrito) para publicação e estão sequencialmente dispostos abaixo. Os itens Introdução, Materiais e Métodos, Resultados, Discussão e Referências Bibliográficas dos artigos estão organizados de forma a facilitar sua leitura e de acordo com as recomendações dos periódicos científicos aos quais foram submetidos.

3.1 Protocolo 1 (Artigo)

N-Acetyl Cysteine Decreases Mice Brain Acetyl Cholinesterase Activity: An in Vitro Kinetic Study

Michael da Costa, Jamile Bernardi, Lidiane Costa, Tiago Fiuza, Ricardo Brandão e Maria Ester Pereira.

Enzyme Engineering 5:135.
doi: 10.4172/2329-6674.1000135

N-Acetylcysteine Decrease Mice Brain Acetylcholinesterase: An *In Vitro* Kinetic Study

Michael da Costa[1], Jamile Bernardi[1], Lidiane Costa[1], Tiago Fiuza[1], Ricardo Brandão[2], Maria E. Pereira[1*].

[1]Department of Biochemistry and Molecular Biology, Laboratory of Biochemistry and Toxicology, Center of Natural and Exact Sciences, Federal University of Santa Maria, Santa Maria, RS, Brazil

[2]Department of Pharmaceutical Sciences, Center of Health Sciences, Federal University of Pernambuco, Recife, PE, Brazil

[*]Correspondence should be sent to:

Maria Ester Pereira

[1]Department of Biochemistry and Molecular Biology, Center of Natural and Exact Sciences, Federal University of Santa Maria, Santa Maria, RS, Brazil. CEP: 97105-900, RS, Brazil.

Phone: 55-55-3220-8799

FAX: 55-55-3220-8978

E-mail: pereirame@yahoo.com

Abstract

Acetylcholinesterase (AChE) is an enzyme responsible for acetylcholine (ACh) neurotransmitter hydrolysis. The enzyme inhibition is clinically used against neurodegenerative diseases like Alzheimer´s (AD). Ellman's method is the most common AChE determination assay used. In this study, we performed a kinetic *in vitro* study of NAC on mice brain AChE activity as well as some studies with the aim to improve the methodology based on NAC proprieties. The ideal 5,5´-dithio-bis-2-nitrobenzoic acid (DTNB) concentration was of 0.3 mM and ideal medium pH = 7.4. AChE demonstrated linear activity for all acetylthiocholine iodide (ATCh) tested concentrations (0.025 - 0.450 mM). To avoid any DTNB-NAC interaction interference, 60 sec were applied to the assay method, so-called stabilization time. No differences in K_m (mM) for all NAC tested concentrations were observed. At the NAC concentrations started from 75 µM significant differences in V_{max} were achieved, characterizing a non-competitive type of AChE activity inhibition induced by NAC. We conclude that NAC decreased AChE activity *in vitro* and the Ellman method is reliable for the analysis of AChE activity when inhibited by compounds which contain SH groups.

Key words: Ellman; methodology; non-competitive.

1. Introduction

Acetylcholinesterase enzyme (AChE; EC 3.1.1.7) is a large glycoprotein whose basic chemistry was worked out in the 1960s by Lawler, Leuzinger and Baker [1]. AChE belongs to the family of hydrolases whose active site is characterized by a catalytic coordinated triad of three essential amino acids: histidine, serine, and glutamic acid [2,3]. This enzyme is one of the best studied of all enzymes from the point of view of its mechanism of action, the nature of its active site, its distribution and localization in tissue, and its physiologic functions [1]. It is mainly found at neuromuscular junctions and cholinergic synapses in the central nervous system (CNS), where its activity terminates the synaptic transmission [4]. AChE has a very high catalytic activity; each molecule of AChE degrades approximately 25,000 molecules of the neurotransmitter acetylcholine (ACh) per second into choline and acetic acid. At the CNS level the produced choline is transported back into the nerve terminals which reuse it in synthesizing new ACh molecules [5-7].

AChE activity can be inhibited by a variety of chemicals, such as organophosphates (OP), carbamates and nerve agents [8] leading to ACh accumulation, resulting in cholinergic receptors over-stimulation and disturbance of many body functions and finally in respiratory arrest and death [9]. Nevertheless, others AChE inhibitors such as physostigmine, pyridostigmine, procycline, donepezil, galantamine, huperezine [10], tacrine and rivastigmine [11] are clinically used against OP poisoning and neurodegenerative diseases like Alzheimer´s (AD). However, all these inhibitors differ in bioavailability, half-life and effectiveness.

N-acetylcysteine (NAC) is a thiol (SH) containing antioxidant, which has been investigated for many pathologies treatment, such as liver failure, inflammation, nephropathy and brain disorders [12]. It is a naturally occurring compound found in several vegetables, including garlic, onion [13], peppers and asparagus [14]. This compound is rapidly absorbed after oral administration in both wild animals and humans [15]. The greatest plasma concentration is reached 2-3 h after administration [16]. NAC has been widely used as an *in vitro* and *in vivo* antioxidant in several experiments [17]. NAC exerts its antioxidant action by facilitating reduced glutathione biosynthesis and scavenging the reactive oxygen species formed during oxidative stress [18]. Many are the reports of beneficial effects of NAC such as renoprotective [19], antiangiogenic [20], anticancer [21], hepatoprotective [22], antifibrotic [23], as chelating agent in metal poisoning treatment [24], and as neuroprotective [25]. However, few are the reports about NAC direct effects on cerebral AChE activity.

It is well known that the most common assay used in AChE determination is based on Ellman's method, which is a photometric method for determining enzyme activity by following the increase of yellow color produced from thiocholine when it reacts with dithiobisnitrobenzoate ion [26]. In this context, we here performed a kinetic *in vitro* study of NAC on mice brain AChE activity, in the attempt to get access to a method that uses the 5,5´-dithio-bis-2-nitrobenzoic acid (DTNB) as chromogen compound functionality and to better understand the potentially direct effect of NAC on this enzyme activity.

2. Materials and methods

2.1. Animals

Adult male *Swiss albino* mice from our own breeding colony (20 - 30 g) were housed in cages and kept on a 12 h light/dark cycle, at a room temperature of 22 ± 2 °C, with free access to food (Guabi, RS, Brazil) and water. All the experiments here described are in accordance with the standards of the Committee on Care and Use of Experimental Animal Resources, the Federal University of Santa Maria, Brazil (Project process number 088/2014). All efforts were made to minimize the number of animals used and their suffering.

2.2. Chemicals

Acetylthiocholine iodide (98%) (ATCh), 5,5´-dithio-bis-2-nitrobenzoic acid (DTNB), N-acetyl-L-cysteine (98%) (NAC), were purchased from Sigma-Aldrich (São Paulo - Brazil). All other reagents used in the experiments were of analytical grade and of the highest purity.

2.3. Methods

2.3.1. Enzyme source

Six *Swiss albino* mice brains were homogenized 1:10 (w/v) in cold ice phosphate potassium buffer (KPB) 0.1 M, pH = 7.4 with glass/teflon homogenizer. The homogenates were centrifuged at 3000 x *g* for 10 min at 4 ºC to yield the salt soluble fraction (SS). The pellet was re-dissolved in KPB 0.1 M, pH = 7.4 containing 1% Triton-X 100 and centrifuged at 15000 x *g* for 10 min at 4 ºC to yield the detergent soluble

fraction (DS), fraction that was used in further assay. Brain mouse choice as enzyme source was based in the perspective to evaluate NAC effects *in vivo* in futures studies.

2.3.2. Enzyme activity and protein determination

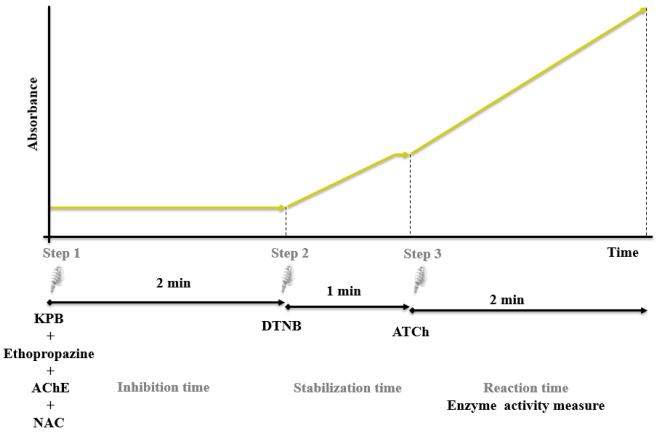
Enzyme activity measurement was performed according to Ellman et al. [26], with some modifications as described in the following steps.

Step 1: In a 96 well plate was added KPB + ethopropazine 20 µM (dissolved in 12 µM hydrochloric acid) and an aliquot of brain DS homogenate (enzyme).

Step 2: DTNB (prepared in KPB) was added at the mixture and allowed to stabilize (stabilization time) at 25 ^{o}C (for stabilization time determination see point 2.3.3.4).

Step 3: Proceeded to the ATCh (prepared in KPB) addition to start enzymatic reaction.

The reaction velocity was measured by increasing absorbance as a result of yellow anion formation (5-thio-2-nitrobenzoate, TNB^{-}) at 412 nm with an absorption coefficient of 0.0136 μM^{-1} cm^{-1}, for 2 min and expressed in $\mu mol.h^{-1}.mg\ prot^{-1}$ (Scheme 1).



Scheme 1. Pippeting procedure and enzymatic activiy measurement.

Protein concentration was measured by the method of Bradford [27], using bovine serum albumin as the standard.

2.3.3. Assay modifications

NAC is a SH containing molecule [22] that can directly interact with the DTNB added to the mixture and so providing false positives results. However, few studies were made with the aim to improve the methodology based in this propriety of NAC, previously to enzyme activity inhibition assay. All experiments were conducted under standard laboratory temperature (25 °C).

2.3.3.1. DTNB concentration and pH determinations

Assays with different concentrations of DTNB were performed. The DTNB final concentrations were of 0.0125, 0.025, 0.05, 0.1, 0.2 and 0.3 mM. After, a pH curve (ranging from 6.8 to 8.0) was performed. From these results, a linearity evaluation was developed, where the 0.3 mM concentration of DTNB and pH = 7.4 were chosen for further analysis.

2.3.3.2. Enzyme activity linearity

For enzyme activity linearity evaluation, enzymatic reaction was performed using different ATCh concentration (0.025, 0.05, 0.1, 0.15 and 0.45 mM).

2.3.3.3. Stabilization time

Immediately after DTNB addition into the medium containing NAC, an absorbance increase is observed resulting of the formation of the NTB^- which ionizes to the NTB^{2-} dianion that has yellow colour. The absorbance increase time for each NAC concentration was measured to determine the required time for the NTB^{2-} formation. For this procedure no enzyme was needed, only NAC was added to a tube containing KPB and 0.3 mM DTNB, the absorbance increase was measured for 2 min.

2.3.4. Enzyme inhibition and kinetics parameters

NAC effect on brain AChE activity was conducted using NAC concentrations of 0, 25, 50, 75, 100, 150 and 200 µM. NAC was added to the mixture containing the KPB and the enzyme and allowed to react for 2 min. Procedure was continued with the addition of DTNB as described in step 2 of section 2.3.2. The kinetic parameters Michaelis-Menten constant (K_m) and maximum velocity (V_{max}), were estimated by assaying the enzyme activity in increasing ATCh concentrations from 0.025 to 0.45 mM.

2.3.5. Time dependence for AChE inhibition and positive control

NAC at the concentration of 169 µM (IC_{50}) was incubated with AChE at different intervals, ranging from 2 to 60 min before the addition of the substrate. After that, the procedure was continued as described in step 2 of section 2.3.2. A positive control (physostigmine 5 µM) was also assayed at the same intervals. The substrate concentration used in this assay was of 0.45 mM.

2.3.5. Statistical analysis

All experimental results are given as the mean (s) ± S.E.M. The statistical analysis was performed using One-way (ANOVA) followed by the Newman-Keuls when appropriate. *p* values < 0.05 were considered significant. The activities were plotted on Michaelis-Menten and Linewever-Burk plots using nonlinear and linear regression respectively. Dixon and Hill plots were performed to determine the inhibition constant (K_i) and to confirm the enzyme inhibition type induced by NAC [28] using software GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego, CA, USA (www.graphpad.com).

3. Results

3.1. DTNB concentration and pH

As shown in Figure 1, AChE activity was lower when used DTNB in a concentration of 0.0125 mM (*p = 0.0002*). No statistical difference was observed in DTNB ranging from 0.025 to 0.3 mM. However, the highest enzyme activity was reached with the concentrations of 0.2 and 0.3 mM. Ideal medium pH was of 7.4 (Fig. 2). No alteration in pH after NAC addition in the medium was observed. AChE activity was linear (r^2 = *0.9996; p = 0.0001*), using DTNB 0.3 mM and pH = 7.4, along the protocol time (Fig. 3).

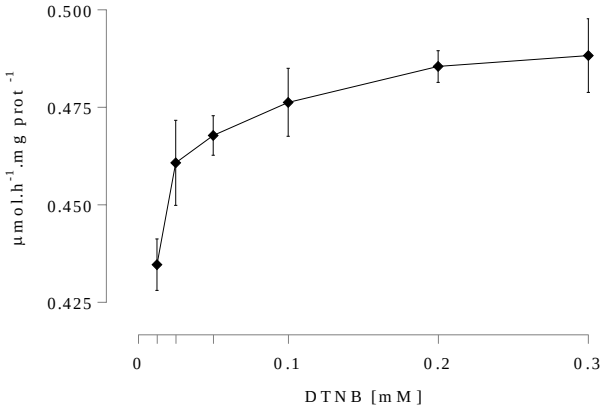


Fig 1. Effect of DTNB concentration (0.0125, 0.025, 0.05, 0.1, 0.2 and 0.3 mM) on AChE activity (µmol.h^{-1}.mg prot^{-1}) from mice brain using 0.45 mM ATCh and pH 7.4 KPB. Data represent the mean ± SEM for 5-6 independent biological replicates measurements.

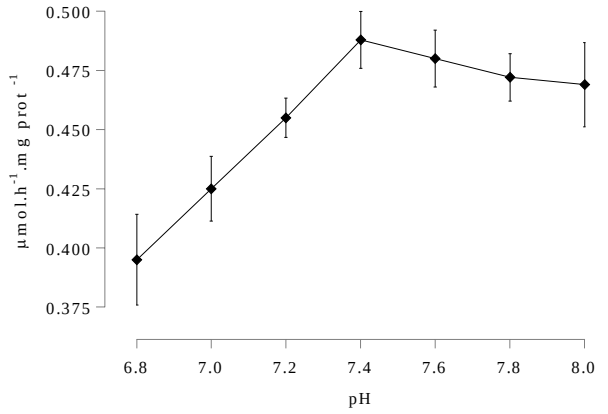


Fig 2. Effect of pH on mice brain AChE activity (µmol.h^{-1}.mg prot^{-1}) using 0.45 mM ATCh and 0.3 mM DTNB. Data represent the mean ± SEM for 5-6 independent biological replicates measurements.

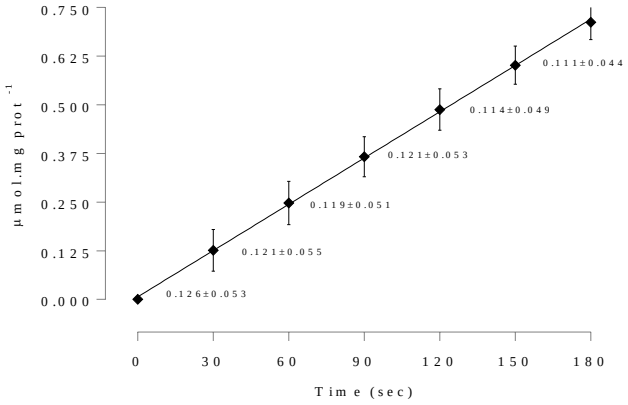


Fig 3. Mice brain AChE activity (µmol.h^{-1}.mg prot^{-1}) linearity (r^2 = *0.9996*) using DTNB 0.3 mM and pH 7.4, along the protocol time. Numbers on graph represent the increase in absorbance every 30 sec (0 - 180 sec). Data represent the mean ± SEM for 5-6 independent biological replicates measurements.

3.2. AChE activity linearity

Linear regression demonstrated AChE linear activity for all ATCh concentrations here tested; 0.025 mM (r^2 = *0.9970*), 0.05 mM (r^2 = *0.9985*), 0.10 mM (r^2 = *0.9975*), 0.15 mM (r^2 = *0.9963*) and 0.45 mM (r^2 = *0.9978*) (Fig. 4) along the 2 min of enzyme activity measurement.

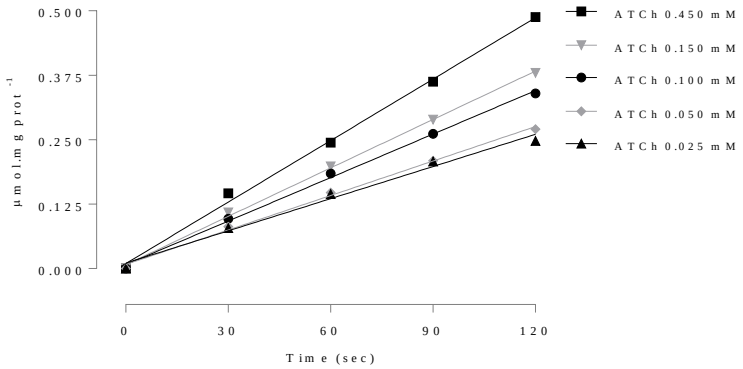


Fig 4. Effect of different ATCh (0.0025, 0.05, 0.10, 0.15 and 0.45 mM) concentration on mice brain AChE activity (µmol.h^{-1}.mg prot^{-1}). Data represent the results for different lines of determination for 5-6 independent biological replicates measurements.

3.3. Stabilization time

Direct time reaction between DTNB and NAC was concentration dependent. For the lower NAC concentration (25 µM) the stabilization time was 17 ± 0.0011 sec, while for the highest concentration (200 µM) was 54 ± 0.0049 sec. In view of these results the

time of 60 sec was concluded to be enough to avoid any interfere of this interaction (Table 1). This 60 sec were applied to the method assay in step 2 (and so called: stabilization time).

Table 1. DTNB-NAC direct reaction time.

NAC [µM]	Stabilization time (sec)
25	17 ± 0.0011
50	20 ± 0.0021
75	25 ± 0.0026
100	35 ± 0.0049
150	47 ± 0.0051
200	54 ± 0.0059

Data are expressed as mean ± S.E.M.

3.4. Enzyme inhibition and kinetics parameters

Statistical analysis demonstrated that no significant difference was observed in K_m (mM) for all NAC tested concentrations (Fig. 5). Nevertheless, significant differences in V_{max} (µmol.h^{-1}.mg prot^{-1}) were observed at started from the NAC concentrations of 75 µM (*p = 0.0369)* (Table 2). Absence of K_m alteration and decrease of V_{max} presented by AChE activity induced by NAC featuring an inhibition of non-competitive type, with a K_i of 147 ± 0.82 µM (Fig. 6). The Hill coefficient (h) was < 1.0 which indicates that once one ligand molecule of NAC is bound to the enzyme, its affinity for other ligand molecules decreases (Fig. 7).

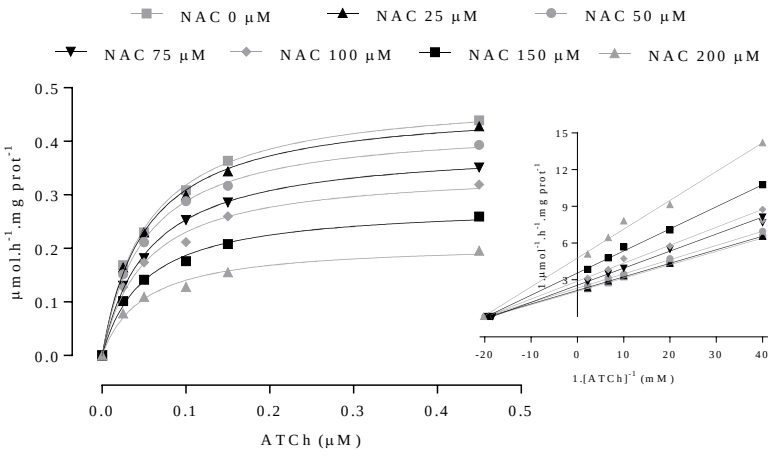


Fig 5. Michaelis-Menten and Linewever Burk plots of NAC inhibitory effect on mice brain AChE activity. Data represent the mean for 5-6 independent biological replicates measurements.

Table 2. NAC effect on kinetic parameters of AChE activity.

NAC [µM]	K_m [mM]	% decrease	V_{max} (µmol.h^{-1}.mg prot^{-1})	% decrease
0	5.33 x 10^{-3} ± 0.0042	-	48.79 x 10^{-3} ± 0.0120	-
25	5.19 x 10^{-3} ± 0.0041	2.63	46.97 x 10-3 ± 0.0116	3.73
50	5.02 x 10^{-3} ± 0.0030	5.81	43.21 x 10-3 ± 0.0080	11.43
75	5.46 x 10^{-3} ± 0.0032	3.19	39.19 x 10-3 ± 0.0072*	19.68
100	5.09 x 10^{-3} ± 0.0073	5.81	34.71 x 10-3 ± 0.0155*	28.86
150	5.09 x 10^{-3} ± 0.0068	5.81	28.21 x 10-3 ± 0.0117*	42.18
200	4.96 x 10^{-3} ± 0.0084	6.94	21.01 x 10-3 ± 0.0109*	56.94

Data are expressed as mean ± S.E.M.

* $p < 0.05$, compared with NAC 0 µM.

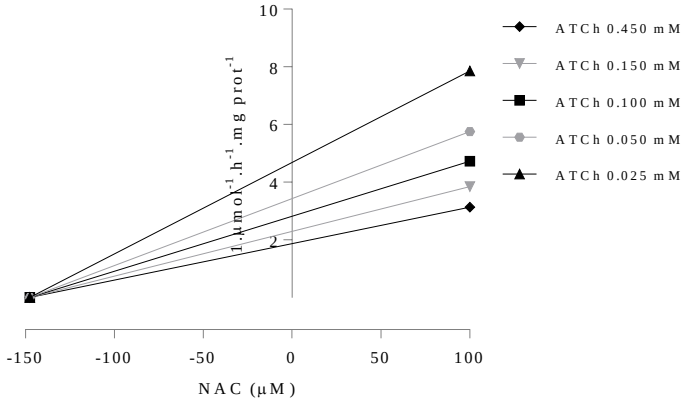


Fig 6. Dixon plot of NAC inhibitory effect on mice brain AChE activity. Data represent the mean for 5-6 independent biological replicates measurements.

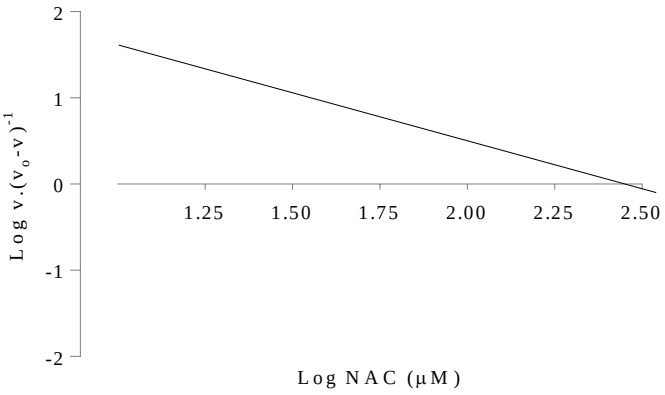


Fig 7. Hill plot of NAC inhibitory effect on mice brain AChE activity, showing the slope of the curve providing apparent dissociation constant. Data represent the mean for 5-6 independent biological replicates measurements.

3.5. Time dependence and positive control

As shown in figure 8 NAC inhibit AChE activity in a manner independent of time. After the incubation with the IC_{50} of NAC the remaining enzyme activity was approximately of 50.33 %, showing no significant difference between 2 and 60 min. The same did not occurs for physostigmine, statistical analysis showed that the incubation time significantly affected the remaining enzyme activity (*F*: 33.47, *p*: 0.0001).

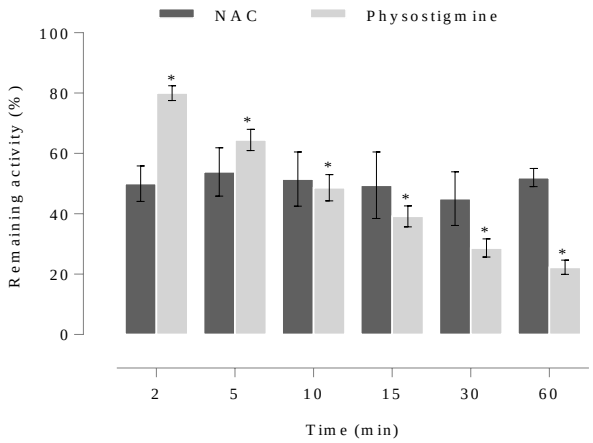


Fig 8. Remaining mice brain AChE activity after NAC incubation at six different times. Data represent the mean ± SEM for 5-6 independent biological replicates measurements. * $p < 0.001$ of One-way ANOVA compared physostigmine at different times.

4. Discussion

This study attempted to determine *in vitro* cerebral AChE activity and the NAC inhibitory effect on this enzyme. The method used was Ellman's using ATCh as alternative substrate and DTNB as color reagent resulting in production of 5-thio-2-nitrobenzoate (TNB^-) that has yellow color due to the shift of electrons to the sulfur atom. This method is particularly limited for testing antidotes against AChE inhibitors or for measuring this enzyme activity in samples of such treated individuals, once some antidotes contain reactive oxime group splitting DTNB and providing false positive reaction [29]. In the same way, NAC can also directly react with DTNB, so in this case, it was mandatory to perform some previous experiments in order to assess the method functionality. We here determinate the DTNB concentration and pH required for a linear enzymatic AChE activity in mouse brain and also added a stabilization time to the method in the attempt to avoid probably false positive reaction (absorbance increase). All ATCh concentrations here tested showed linear activity. Finally, the results here achieved show that NAC behave as a reversible non-competitive AChE inhibitor, by reducing enzyme V_{max} in a concentration dependent way without interfering in the K_m.

The most common assay used in AChE determination is based on Ellman's method. Over the last years' different chromogens have been tested [30], nevertheless, in this work we choose to use DTNB. The present results demonstrated that DTNB at concentration ranging from 0.025 to 0.3 mM were enough to provide similar AChE activity reading (46.08×10^{-3} and 48.83×10^{-3} $\mu mol.h^{-1}.mg\ prot^{-1}$, respectively) along the protocol time. Thus, we here use the 0.3 mM final concentration to avoid any hypothesis of chromogen consumption by directly interact with NAC. In fact, this chromogen has acid pH and can directly interfere with enzyme activity. Here, assays were performed with DTNB solutions in a range from 6.8 to 7.6. As showed by the present results the higher activity was achieved with pH = 7.4. Besides, knowing the ideal pH is critical when working with inhibitors. The enzymes inhibition may be greatly affected by pH, in the irreversible no less than the reversible case. The rate reaction of the inhibitor with the enzyme may be affected by pH because the different ionic forms of the enzyme may react at different rates with the inhibitor, or because the inhibitor ionizes itself [28].

For all ATCh concentrations here used the enzyme activity was linear, thus demonstrating that this decrease was not due to lack of substrate. Moreover, substrate inhibition is a kinetic property of AChE [31]. However, the highest substrate final concentration (ATCh 0.45 mM) tested was based on previously studies of our laboratory [32], which demonstrate to be a non-inhibitory substrate concentration. The 1 min waiting

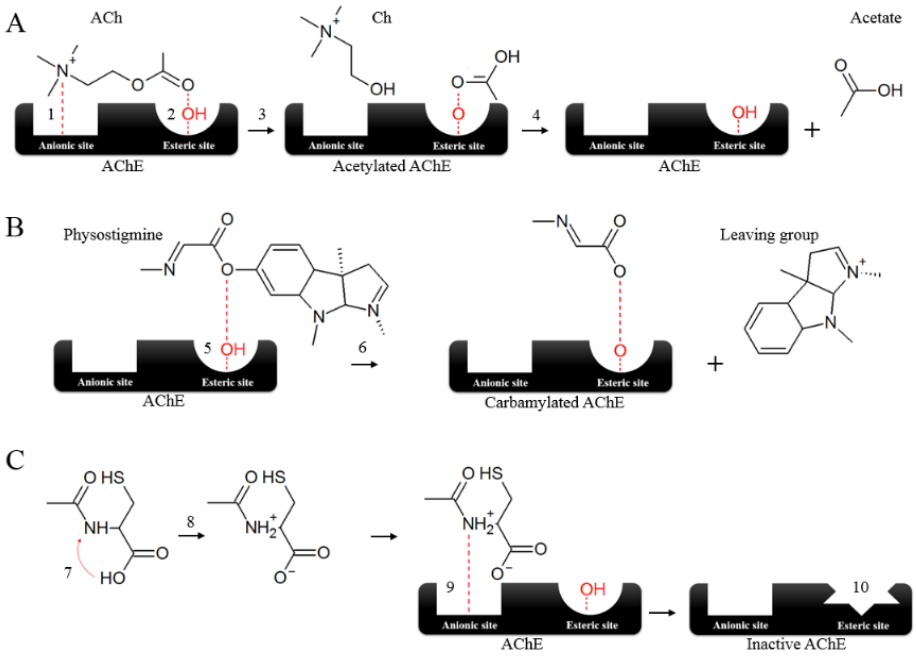
time addition (stabilization time) was established to avoid interference of the direct reaction of DTNB with NAC, which occurs immediately after the DTNB addition in the medium, causing an immediate increase in absorbance. It is a fact that high concentrations of Triton X-100 can decrease AChE activity, however, the concentration used in this work did not have any impact on the enzyme activity decrease and was used only in the attempt to get the total AChE activity in the medium.

Enzyme kinetics is the study of the chemical reactions catalyzed by enzymes. Through this, we can show the catalytic mechanism of an enzyme, its role in metabolism, how its activity is controlled or how a drug or an agonist might interfere in the enzyme activity. Our results showed that NAC can decrease *in vitro* AChE activity of mice brains. In 1966, Dixon and Webb proposed that a single enzyme involved in a main metabolic chain inhibition will render the whole chain inoperative and will have a profound or even fatal effect upon the organism. However, some exceptions to this rule apply [28]. To date, the use of AChE inhibitors is one of the few therapies that show consistent positive results in the AD treatment [33]. Here, NAC at 75 µM significantly decrease *in vitro* AChE activity in approximately 20%, when compared with the normal enzyme activity being the IC_{50} of 169 µM. As can be seen by the V_{max}, the enzyme activity decrease was dependent on the NAC used concentration. However, by varying the substrate concentration the K_m remained unchanged, characteristic of a non-competitive inhibition, where the inhibitor molecule binds to an enzyme somewhere other than the active site reducing the enzyme activity or binds equally well to the enzyme whether it has already bound the substrate.

AChE non-competitive inhibitors are clinically used in AD treatment, like Tacrine [34], Donepezil and Huperzine A [35] to name a few. Besides, despite the countless effects of NAC on different neurological disorders like AD by increase the glutathione levels or free-radical scavenging [36], few are the reports of its effect on cerebral AChE activity. Hill coefficients give information on the number of interacting sites. When this coefficient is smaller than one, do not indicate anticooperative binding but show that at least one ternary complex has enzymatic activity [37]. Here, Hill coefficients was smaller than one indicating a possible allosteric binding, where induced conformational changes cause loss of the active conformation. Such a denaturation mechanism is in stark contrast to the desired specificity of drugs.

NAC is structurally similar to cysteine amino acid which can easily form a dipolar ion so called zwitterion at neutral pH [38]. We here believe that NAC can also form a zwitterion, a molecule with no overall electrical charge, but that contains separate positive and negative parts (N^+ and O^-, respectively). The cationic region (N^+) present in

the NAC zwitterion suffers an electrostatic attraction by the AChE anionic site, inducing a temporally modification in the enzyme esteric site and disabling the neurotransmitter bind (Scheme 2). The fact that NAC inhibitory effect over AChE activity did not vary according to the length of the preincubation period, suggests that the NAC effect is of reversible nature [39] similar to physostigmine, which also reversibly inhibit the AChE, even though that this binds to the active site of the enzyme and not to the anionic one.



Scheme 2. Summarized interaction of ACh (A), physostigmine (B) and NAC (C) with the AChE. In normal conditions the AChE anionic site accommodates the positive quaternary amine of ACh (1) while the acetyl group binds to the serine present in the esteric site (2) yielding an acetylated enzyme (3) and choline. After a rapid hydrolyzes (4) acetate is formed. When physostigmine is add to the medium an electrostatic bond is formed between carbamate oxygen and the serine oxygen which is located in the esteric site (5) and an inactive (carbamylated) enzyme is formed (6) [40]. When NAC is in neutral medium the COOH donates a proton to the NH_2 group (7) and form a zwitterion whit a positive and a negative electrical charge (8), in an internal acid-base reaction. The quaternary amine suffers an electrostatic attraction by the anionic site of the enzyme (9), this bind causes a structural modification in the active site of the enzyme yielding to an enzyme inactivation (10).

5 Conclusion

Thus, we conclude that the Ellman method is reliable for the analysis of AChE activity when inhibited by compounds which contain SH groups. NAC inhibited non-competitively the enzymatic activity of AChE, been considered as an AChE inhibitor for further *in vivo* studies.

Conflict of interest statement

All the authors declare that there are no conflicts of interest.

Acknowledgements

The financial support by FAPERGS, CAPES, and CNPq is grate fully acknowledged. Additional support was given by CNPq/FAPERGS/DECIT/SCTIE-MS/PRONEM #11/2029-1.

References

[1] S. Brimijoin, Molecular forms of acetylcholinesterase in brain, nerve and muscle: nature, localization and dynamics. Progr. Neurobiol. 21 (1983) 291-322.

[2] A. Krasinski, Z. Radic, R. Manetsc, J. Raushel, P. Taylor, K.B. Sharpless, H.C. Kolb, In situ selection of lead compounds by click chemistry: target-guided optimization of acetylcholinesterase inhibitors. J. Am. Chem. Soc. 127 (2005) 6686-6692.

[3] R. Manetsch, A. Krasinski, Z. Radic, J. Raushel, P. Taylor, K.B. Sharpless, H.C. Kolb, In situ click chemistry: enzyme inhibitors made to their own specifications. J. Am. Chem. Soc. 126 (2004) 12809-12818.

[4] P. Norouzi, M. Pirali-Hamedani, M.R. Ganjali, F.A. Faridbod, A novel acetyl cholinesterase biosensor based on chitosan-gold nanoparticles film for determination of monocrotophos using FFT continuous cyclic voltammetry. Int. J. Electrochem. Sci. 5 (2010) 1434-1446.

[5] V. Whittaker, The Contribution of drugs and toxins to understanding of cholinergic function. Trends. Pharmacol. Sci. 11 (1990) 8-13.

[6] D. Purves, G.J. Augustine, D. Fitzpatrick, W.C. Hall, A.S. LaMantia, J.O. McNamara, L.E. White, Neuroscience, fourth ed., Sinauer Associates, Sunderland: MA; 2008.

[7] M. Pohanka, Alpha7 Nicotinic Acetylcholine receptor is a target in pharmacology and toxicology. Int. J. Mol. Sci. 13 (2012) 2219-2238.

[8] V.C. Moser, S. Padilla, Esterase metabolism of cholinesterase inhibitors using rat liver in vitro. Toxicology 281 (2011) 56-62.

[9] F. Worek, U. Mast, D. Kiderlen, C. Diepold, P. Eyer, Improved determination of acetylcholinesterase activity in human whole blood. Clin. Chim. Acta. 288 (1999) 73-90.

[10] T. Myhrer, S. Enger, P. Aas, Behavioral side effects in rats treated with acetylcholinesterase inhibitors suggested used as prophylatic against nerve agent. Pharmacol. Biochem. Behav. 95 (2010) 338-343.

[11] R.J. Polinsky, Clinical pharmacology of rivastigmine: A new-generation acetylcholinesterase inhibitor for the treatment of Alzheimer´s disease. Clin. Ther. 20 (1998) 635-47.

[12] W. Chen, N. Ercal, T. Huynh, A. Volkov, C.C. Chusuei, Characterizing N-acetylcysteine (NAC) and N-acetylcysteine amide (NACA) binding for lead poisoning treatment. J. Colloid. Interface. Sci. 371 (2012) 144-149.

[13] Y.C. Hsu, Y.T. Chiu, C.C. Wu, C.F. Cheng, Y.L. Lin, Y.T. Huang, Antifibrotic effects of tetrandrine on hepatic stellate cells and rats with liver fibrosis. J. Gastroenterol. Hepatol. 22 (2007) 99-111.

[14] O. Demirkol, C. Adams, N. Ercal, Biologically important thiols in various vegetables and fruits. J. Agric. Food Chem. 52 (2004) 8151-8154.

[15] L. Decaro, A. Ghizzi, R. Costa, Pharmacokinetics and bioavailability of oral acetylcysteine in healthy volunteers. Arzneimittelforschung 39 (1989) 382-385.

[16] C. Babiloni, C. Del Percio, R. Bordet, J.L. Bourriez, M. Bentivoglio, P. Payoux, P. Derambure, S. Dix, F. Infarinato, R. Lizio, A.I. Triggiani, J.C. Richardson, P.M. Rossini, Effects of acetylcholinesterase inhibitors and memantine on resting-state electroencephalographic rhythms in Alzheimer's disease patients. Clin. Neurophysiol. 124 (2013) 837-850.

[17] M. Carvalho, F. Remião, N. Milhazes, F. Borges, E. Fernandes, F. Carvalho, M.L. Bastos, The toxicity of N-methyl-amethyldopamine to freshly isolated rat hepatocytes is prevented by ascorbic acid and N-acetylcysteine. Toxicology 200 (2004) 193-203.

[18] K. Ocal, D. Avlan, I. Cinel, A. Unlu, C. Ozturk, F. Yaylak, M. Dirlik, H. Camdeviren, S. Aydin, N-acetylcysteine on oxidative stress in intestine and bacterial translocation after thermal injury. Burns. 30 (2004) 778-784.

[19] S.N. Heyman, M. Goldfarb, A. Shina, F. Karmeli, S. Rosen, N-acetylcysteine ameliorates renal microcirculation: studies in rats. Kidney Int. 63 (2003) 634-641.

[20] A. Albini, M. Morini, F.D. Agostini, N. Ferrari, F. Campelli, G. Arena, D.M. Noonan, C. Pesce, S.D. Flora, Inhibition of angiogenesis driven Kaposi's, sarcoma tumor growth in nude mice by oral N-acetylcysteine. Cancer Res. 61 (2001) 8171-8178.

[21] J.H. Wang, J.W. Shin, J.Y. Son, J.Y. Cho, C.G. Son, Antifibrotic effects of CGX, a traditional herbal formula, and its mechanisms in rats. J. Ethanopharmacol. 127 (2010) 534-542.

[22] P. Sathish, V. Paramasivan, V. Palani, K. Sivanesan, N-acetylcysteine attenuates dimethylnitrosamine induced oxidative stress in rats. Euro. J. Pharmacol. 654 (2011) 181-186.

[23] G.P. Filho, C. Ferriera, A. Schwengber, C. Marroni, C. Zettler, N. Marroni, Role of N-acetylcysteine on fibrosis and oxidative stress in cirrhotic rats. Arq. Gastrenterol. 45 (2008) 156-162.

[24] M. Modi, R.K. Kaul, G.M. Kannan, S.J.S. Flora, Co-administration of zinc and N-acetylcysteine prevents arsenic induced tissue oxidative stress in male rats. J. Trace Elem. Med. Biol. 20 (2006) 197-204.

[25] S. Eskiocak, S. Altaner, S. Bayir, E. Cakir, The effect of N-acetylcysteine on brain tissue of rats fed with high cholesterol diet. Turk. J. Biochem. 33 (2008) 58-63.

[26] G.L. Ellman, K.D. Courtney, Jr.V. Andres, R.M. Featherstone, A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7 (1961) 88-95.

[27] M.M. Bradford A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principles of protein-dye binding. Anal. Biochem. 72 (1976) 248-254.

[28] M. Dixon, E.C. Webb, Enzymes. Ballantyne and Co Ltda. Sec Ed. London: Great Britain; 1966.

[29] G. Sinko, M. Calic, A. Bosak, Z. Kovarik, Limitation of the Ellman method: Cholinesterase activity measurement in the presence of oximes. Anal. Biochem. 370 (2007) 223-227.

[30] M. Pohanka, M. Hrabinova, K. Kuca, J. Simonato, Assessment of Acetylcholinesterase Activity Using Indoxylacetate and Comparison with the Standard Ellman's Method. Int. J. Mol. Sci. 12 (2011) 2631-2340.

[31] H.C. Froede, I.B. Wilson, H. Kaufman, Acetylcholinesterase: Theory of Noncompetitive Inhibition. Arch. Biochem. Biophys. 247 (1986) 420-423.

[32] M.D. Costa, M.L. Freitas, F.A.A. Soares, V.S. Carratu, R. Brandão, Potential of two new oximes in reactivate human acetylcholinesterase and butyrylcholinesterase inhibited by organophosphate compound: An in vitro study. Toxicol. in vitro. 25 (2011) 2120-2123.

[33] L. Bonanomi, A. Gazzaniga, Toxicological pharmacokinetic and metabolic studies on acetylcysteine. Euro. J. Respir. Dis. Suppl. 61 (1980) 45-51.

[34] M. Holden, C. Kelly, Use of Cholinesterase Inhibitors in Dementia. Adv. Psych. Treat. 8 (2002) 89-96.

[35] B.S. Wang, H. Wang, Z.H. Wei, Y.Y. Song, L. Zhang, H.Z. Chen, Efficacy and safety of natural acetylcholinesterase inhibitor huperezine A in the treatment of Alzheimer's disease: an updated meta-analysis. J. Neural. Transm. 116 (2009) 457-465.

[36] R.B. Shahripour, M.R. Harrigan, A.V. Alexandrov, N-acetylcysteine (NAC) in neurological disorders: mechanism of action and therapeutic opportunities. Brain Behav. 4 (2014) 108-122.

[37] H. Prinz, Hill coefficients, dose–response curves and allosteric mechanisms. J. Chem. Biol. 3 (2010) 37-44.

[38] M.J. Betts, R.B. Russell, Amino Acid Properties and Consequences of Substitutions, In: Bioinformatics for Geneticists. Wiley & Sons Ltda. 2003. Chap. 14.

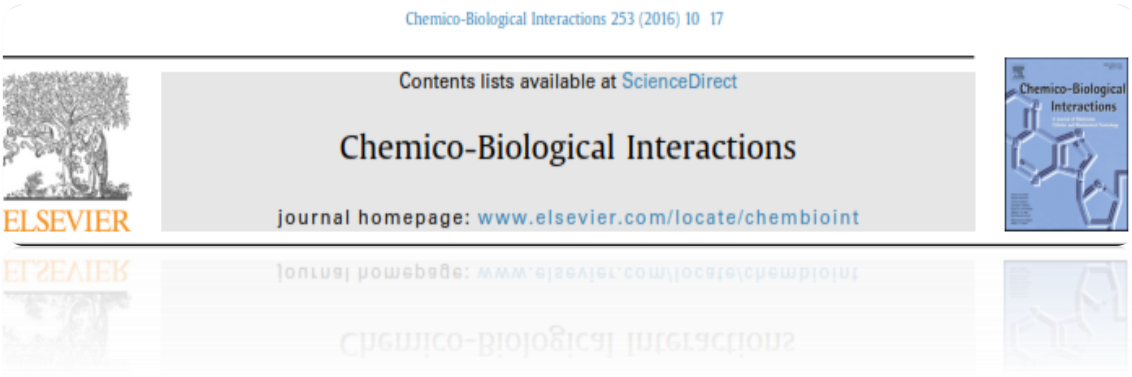
[39] R. Dowson, Reversibility of the inhibition of acetylcholinesterase by tancrine. Neurosci. Lett. 11 (1990) 85-87.

[40] C. Timchalk, Physiologically based pharmacokinetic modeling of organophosphorus and carbamate pesticides. In Toxicology of Organophosphate and Carbamate Compounds, Gupta RC (ed.). Academic Press/Elsevier, Amsterdam 2006, pp. 103-125.

3.2 Protocolo 2 (Artigo)

N-acetylcysteine protects memory decline induced by streptozotocin in mice

Michael da Costa, Jamile Bernardi, Tiago Fiuza, Lidiane Costa, Ricardo Brandão e Maria Ester Pereira.



Chemico-Biological Interactions 253 (2016) 10-17
doi: org/10.1016/j.cbi.2016.04.026

N-acetylcysteine protects memory decline induced by streptozotocin in mice

Michael da Costa[1], Jamile Bernardi[1], Tiago Fiuza[1], Lidiane Costa[1], Ricardo Brandão[2], Maria E. Pereira[1*].

[1]Department of Biochemistry and Molecular Biology, Laboratory of Biochemistry and Toxicology, Center of Natural and Exact Sciences, Federal University of Santa Maria, UFSM Campus, Santa Maria, RS, Brazil

[2]Department of Pharmaceutical Sciences, Center of Health Sciences, Federal University of Pernambuco, UFPE Campus, Recife, PE, Brazil

[*]Correspondence should be sent to:

Maria Ester Pereira

[1]Department Biochemistry and Molecular Biology, Center of Natural and Exact Sciences, Federal University of Santa Maria, Santa Maria, RS, Brazil. CEP: 97105-900, RS, Brazil.

Phone: 55-55-3220-8799

FAX: 55-55-3220-8978

E-mail: pereirame@yahoo.com.br

Abstract

Alzheimer's disease (AD) is a neurodegenerative disease characterized by cognitive impairment, associated with a reduced concentration of acetylcholine (ACh) in brain cortex and hippocampus. Recently we reported that the N-acetylcysteine (NAC) decreases brain acetylcholinesterase (AChE) activity *in vitro.* Thus, the aim of the current study was to investigate the effect of NAC against streptozotocin (STZ) induced AD in mice. Mice were divided into four groups: I) Sham, II) NAC, III) STZ and IV) NAC+STZ. Animals were daily treated with NAC (50 mg/kg/day, p.o.) for nine consecutive days and with STZ (2.5 mg/kg i.c.v.) at the first and third days. Step down passive avoidance (SDPA, days 7-8) and Morris water maze (MWM, days 6-9) task were assessed to evaluate learning and memory. On the tenth day animals were euthanized for AChE and butyrylcholinesterase (BChE) and ACh, energy-rich phosphate and brain glucose uptake levels evaluations. A learning and memory impairment was observed in SDPA and MWM in those animals that receive STZ. Nevertheless, the same was not observed in those animals that also received NAC. Brain cortex and hippocampus AChE and hippocampus BChE activities increase induced by STZ were also prevented by NAC treatment. The STZ induced a brain energy metabolism imbalance, decreasing adenosine triphosphate and increasing adenosine levels. The glucose uptake decrease in hippocampus was prevented by NAC. In conclusion, NAC treatment prevented the cognitive disturbance, by restoring the cholinergic system and brain energy metabolism disorders. NAC could modulate cholinergic imbalance without causing any changes *per se* in the same.

Key words: Cognition; cholinesterases; acetylcholine; behavioral.

1. Introduction

Cognitive impairment is a broad term to describe any characteristic that acts as a barrier to the process of cognition [1]. Some of the causes of cognitive impairment in humans are neurodegenerative diseases such as Alzheimer's disease (AD), comprising 50-70% of all cases [2]. AD is characterized by a progressive loss of memory, thinking, learning capacity and judgment [3]. This neurodegenerative disease is coupled to mitochondrial dysfunction, which leads to an impaired energy metabolism and consequent activation of proapoptotic pathways signaling [4]. The death of neurons, particularly in the cerebral cortex and hippocampus, have also been related to AD [5]. Severe alterations in glucose metabolism are also reported in all forms of sporadic dementia resulting from AD, since the use of glucose and the levels of energy-rich phosphate compounds are reduced in brain tissue from AD patients. Disruption in the control of neuronal glucose metabolism, cosequence of the failure in signal transduction triggered by insulin, is also observed in the early AD pathogenesis [6]. This neuronal glucose metabolism controls the release of neurotransmitters at synapses, activating pathways associated with learning and long-term memory [7].

Streptozotocin (STZ) is a naturally occurring nitrosamine, which has been widely used as diabetes inducing in experimental animals. However, an intracerebroventricular (i.c.v.) injection of STZ in a sub diabetogenic dose has been reported as a model of AD, leading to a memory deficit [8]. This model is associated with disorders in the glucose use and consequent changes in energy metabolism [9], leading to cognitive dysfunctions by reducing the synthesis of adenosine triphosphate (ATP) and acetyl coenzyme A (acetyl-CoA). These changes result in a cholinergic dysfunction characterized by the choline acetyltransferase (ChAT; EC 2.3.1.6) activity inhibition and the acetylcholinesterase (AChE; EC 3.1.1.7) and butyrylcholinesterase (BChE; EC 3.1.1.8) activities increase in the nervous system [10,11].

AChE along with BChE belong to the hydrolases family. In the brain, AChE is primarily associated neurones [12], while BChE is mainly located in glial cells [13]. The main function of AChE is the (acetylcholine) ACh hydrolysis, this neurotransmitter is synthesized from choline and acetyl-CoA in the presynaptic neuron and released into the synaptic space where it will bind to the pre and postsynaptic muscarinic and nicotinic receptors, stimulating both excitatory and inhibitory transmission [14]. BChE is less substrate-specific [15]. Nevertheless, both enzymes are able to hydrolyze ACh at a rate of > 10000 molecules per second [16]. Thus, these enzymes are strongly involved in cognitive learning and memory function [17,18]. In regard of the AD cholinergic

hypothesis, which is based on the decline in cognitive function due to loss of cholinergic neurotransmission mainly in brain cortex and hippocampus, along the years the focus of drug development for AD has been on strategies that enhance central cholinergic function. Amongst them, the use of AChE inhibitors seems to be the one with best results [14].

N-acetylcystein (NAC) is a derivative of the amino acid cysteine molecule clinically used in respiratory diseases, acetaminophen poisoning and renal disease treatments [19,20]. This molecule also have anti-inflammatory, anti-apoptotic and pro-neurogenic properties [21]. Knowing this, the use of NAC as neuroprotective in psychiatric and neuroprogressive disorders [22] and in cognitively related treatments [23], have been propose. Moreover, the neuroprotective effect of NAC against STZ-induced memory dysfunction in rats after three weeks treatment has been reported [24]. Based on the above considerations, the purpose of the present study was to investigate the potential neuroprotective effect of NAC on the cognitive deficit induced by i.c.v. STZ in mice.

2. Materials and methods

2.1. Animals

Experiments were conducted using male *Swiss* mice (25 - 30 g) about two months old. Animals were maintained at 22 ± 2 °C with free access to water and food under a 12 h: 12 h light/dark cycle with lights on at 07:00. All manipulations were carried out between 08:00 and 16:00. Animals were used according to the guidance of the Committee on Care and Use of Experimental Animal Resources, the Federal University of Santa Maria, Brazil (Process Nº 088/2015) and the ARRIVE guidelines. All efforts were made to minimize animals' suffering and to reduce the number of animals used in the experiments.

2.2. Chemicals

NAC, STZ, adenosine (ADO), adenosine 5-monophosphate (AMP), adenosine 5-diphosphate (ADP), adenosine 5-triphosphate (ATP), acetylcholine (ACh), acetylthiocholine (ATCh), butyrylthiocholine (BTCh), acetylcholinesterase (AChE), 5,5´-dithiobis-(2-nitrobenzoic acid) (DTNB), choline oxidase (ChOx), ethopropazine, ethylenediaminetetraacetic acid (EDTA)and azinobis-3-ethyl-benzothiazoline-6-sulfonic

acid (ABTS) were obtained from Sigma Chemical (St. Louis, MO, USA). All other chemicals were obtained in an analytical grade or from standard commercial suppliers.

2.3. Experimental procedure

2.3.1. Memory impairment induction

Animals were divided into four groups (8 animals per group): I) Sham; II) NAC, III) STZ and IV) NAC+STZ. For treatment one: animals belonging to groups II and IV were treated p.o., through oral gavage, with a single NAC dose of 50 mg/kg body weight dissolved in distilled water each day, during nine consecutive days. NAC dose which showed no toxicity to animals was chosen based on a curve dose ranging from 0 to 150 mg/kg (data not shown). Animals belonging to groups I and III received distilled water instead. For treatment two: animals belonging to groups III and IV received a intracerebroventricular (i.c.v.) injection of STZ 2.5 mg/kg body weight dissolved in artificial cerebral spinal fluid [aCSF (mmol/L): 147.0 NaCl; 2.9 KCl; 1.6 $MgCl_2$, 1.7 $CaCl_2$ and 2.2 $C_6H_{12}O_6$], pH = 7.4 in a fixed volume of 5 µl at the first and third days. Animals from groups I and II received only aCSF (5 µl/site) [25]. The i.c.v. administration of STZ (or aCSF) was performed by a “free hand” protocol with the bregma fissure as a reference point based on the previous published method [26,27]. Briefly, the animals were anesthetized with isoflurane and grasped firmly by the loose skin behind the head, the skin was pulled tight and a hypodermic needle (27 gauge) attached to a 25 µl Hamilton microsyringe was inserted perpendicularly through the skull into the brain. The site of injection was 2 mm either side of the midline on a line drawn through the anterior base of the ears; the depth of insertion of the needle was about 3 mm. To find out if the STZ was administered exactly into the cerebral ventricle, mice were killed by cervical dislocation and the brains were dissected and macroscopically examined.

2.4. Behavioral tests

2.4.1. Open field test

Spontaneous locomotor activity was measured in the open field (OF) test [28] at the day 6, previously to the memory task. The floor of the open field was divided into nine squares. Each animal was placed individually in the center of the arena, and the number of segments crossed (four-paw criterion) and rearings were recorded in a 5 min session.

2.4.2. Step-down passive avoidance task

The step-down passive avoidance (SDPA) task has been used to study nonspatial long-term memory [29]. The apparatus consisted of a single box where the floor was made of a metal grid connected to a shock scrambler. A safe platform was also placed in the box. During the training session (acquisition trial, day 7), the mouse was placed on the platform, when the animal stepped down and placed their four paws on the grid floor, an electric shock (0.5 mA) was delivered for 2 sec. Some sec later (~5), the mouse was removed from the step-down passive avoidance apparatus and returned to its home cage. The retention trial was performed 24 h after training (day 8). Each mouse was placed again on the platform, and the transfer latency time (i.e., time took to step down from the platform) was measured in the same way as in the acquisition trial, nevertheless, shock was not delivered and the transfer latency time was recorded to a maximum of 5 min. The criterion for learning was taken as an increase in the transfer latency time on retention (day 8) trial as compared to the acquisition (day 7) trial. Therefore, short transfer latencies indicate poor retention.

2.4.3. Morris water maze task

Spatial learning and memory were assessed using the Morris water maze (MWM) task, according to the method of Morris [30]. The maze consisted of a basin (diameter: 180 cm, wall height: 40 cm) made of black plastic and filled with water (25 ± 2 °C) at a height of 30 cm. The pool was placed in a room with several extra-maze visual cues. For the acquisition phase, the mouse was submitted to four trials each day [starting in the north (N), than east (E), than south (S) and last west (W)] for three consecutive days (days 6, 7 and 8). The escape platform was hidden 0.5 cm below water level in the middle of the northwest (NW) quadrant. The mouse remained on the platform for at least 40 sec after each trial. Whenever the mouse failed to reach the escape platform within the 1 min cutoff period, it was retrieved from the water and placed on the platform for 40 sec. The time spent in each quadrant, the thigmotactic behavior and the latencies to reach the platform were calculated as the mean of total time spent in four trials of each day. Twenty-four hours after the acquisition phase (day 9), a probe trial was conducted by removing the platform and placing the mouse next to and facing the SE side. The latency to reach the platform site and the number of crossings over the former platform site were measured for a single 1 min trial.

2.5. Biochemical assays

2.5.1. Tissue collection

Twenty-four hours after the last behavioral test (day 10), the animals were anesthetized with isoflurane. Subsequently, mice were euthanized by blood extraction through cardiac puncture and the cerebral tissue was removed and dissected into different regions (brain cortex, hippocampus and striatum) under board certified personal guidance.

2.5.2. AChE and BChE activities

Enzyme activities were carried out according to the method of Ellman et al. [31]. Cerebral tissue (brain cortex, hippocampus and striatum) samples were homogenized in 0.1 mol/L potassium phosphate buffer (KPB), pH = 7.4 in a 1:10 (w/v) ratio, containing 1% Triton X-100. The homogenate was centrifuged at 12000 × g for 10 min to yield the low-speed supernatant (S_1) fraction that was used in the assay. Then 0.3 mmol/L DTNB was added and the enzymatic reaction was initiated by addition of 0.45 mmol/L of ATCh and BTCh as substrate for AChE and BChE, respectively. For AChE activity, 0.02 mmol/L ethopropazine hydrochloride was also added to avoid BChE interference. Enzyme activities were spectrophotometrically measured at 412 nm for 3 min. Results were expressed in µmol ACTh/h/mg protein and µmol BCTh/h/mg protein for AChE and BChE, respectively. Total protein concentration was measured by the method of Bradford [32] using bovine serum albumin as the standard.

2.5.3. ACh levels

ACh concentration in the cerebral tissue (brain cortex, hippocampus and striatum) was evaluated based on choline oxidase (ChOx) activity modify method [33]. The tissues samples were homogenized in 0.1 mol/L KPB, pH = 7.4 in a 1:10 (w/v) ratio and heated in boiling water for 3 min and placed in ice. After that, the homogenate was centrifuged at 12000 × g for 10 min to yield the low-speed supernatant fraction (S_1) which was used in the assay. Then, the S_1 was incubated at 37 °C for 30 min with 0.025 U/mL purified AChE and 0.01 U/mL ChOx prepared in 0.1 mol/L KPB, pH = 7.4. After, 0.5 mmol/L $KMnO_4$ (chromogen) solution was added to the mixture and incubated at 37 °C. The decrease in absorbance after 30 min was measured at 490 nm. ACh was used as standard with the final concentration ranging from 0 - 375 µmol/L. Results were expressed as nmol ACh/mg tissue.

2.5.4. Energy-rich phosphates levels

The energy-rich phosphate (ATP, ADP, AMP and ADO) levels in mice total brain were determined by the HPLC-UV detection modified method of Özogul et al. [34]. Briefly, brain samples were homogenized in 0.6 mmol/L $HClO_4$ and centrifuged at 2400 × g at 4 °C for 10 min. The supernatant fraction (S_1) was neutralized to pH = 6.0 - 6.5 with 1 mol/L KOH. The neutralized S_1 were kept on ice for 30 min to make sure the total precipitation of the K crystals. After that, they were filtered through a membrane (0.45 µm pore size; Millipore) before the injection into the HPLC instrument. The analytical column was a 5 µm particle and 100 Å pore size Phenomenex ODS2 C^{18} reverse-phase column (4.6 × 250 mm, Allcrom, Brazil). The mobile phase was 0.04 mol/L KH_2PO_4 and 0.06 mol/L K_2HPO_4 dissolved in purified distilled water and adjusted to pH = 7.0 with 0.1 mol/L KOH. The HPLC analysis was performed under isocratic conditions at a flow rate of 1 mL/min and with the UV detector set at 257 nm. Results were expressed as nmol/g tissue.

2.5.5. Glucose uptake

Glucose uptake was determined based on a previous study [35] with some modifications. Briefly, slices of cerebral tissue (brain cortex, hippocampus and striatum) were incubated for 30 min at 37 ºC with 1 ml of Krebs-Henseleit Bicarbonate buffer [KHB (mmol/L): 118 NaCl, 25 $NaHCO_3$, 4.8 KCl, 1.2 KH_2PO_4, 1.2 $MgCl_2$ and 11 $C_6H_{12}O_6$], pH = 7.4. The glucose concentration of each sample was spectrophotometrically determined at initial time and after 30 min of incubation. The amount of the brain glucose uptake was calculated as the difference of the glucose concentration in each sample between times (final time - initial time). Results were expressed as µg of glucose uptake/mg of tissue.

2.6. Data presentation and statistical analysis

All experiments results are given as the mean (s) ± S.E.M. Statistical analysis for behavioural and *in vivo* tests were performed using two-way ANOVA followed by Duncan's post-hoc test when apropriated. Main effects of first-order are presented only when interaction was not significant. Values of $p < 0.05$ were considered statistically significant. Calculations were performed using software STATISTICA 10 Copyright© Stat Soft Inc. Tulsa, OK - USA (www.statsoft.com).

3. Results

3.1. Behavioral tests

3.1.1. Open field

Mice performance in the OF test is presented in Table 1. No effect on spontaneous nor in exploratory activity was observed by any of the treatments.

3.1.2. Step-down passive avoidance

Mice performance in the SDPA task is presented in Table 1. No difference among groups in the transfer latency time in the acquisition phase was observed. Statistical analysis of the transfer latency time in retention trial showed a significant NAC × STZ interaction ($F_{1,28} = 6.358$; $p = 0.018$). Post-hoc comparisons revealed that STZ group presented lower transfer latency time (~56%) than Sham group; the NAC treatment prevented this impairment.

Table 1. Effects of NAC administration on spontaneous locomotor and exploratory activity in the open field-test (OF) and step-down passive avoidance test (SDPA) in mice that received i.c.v. injection of STZ.

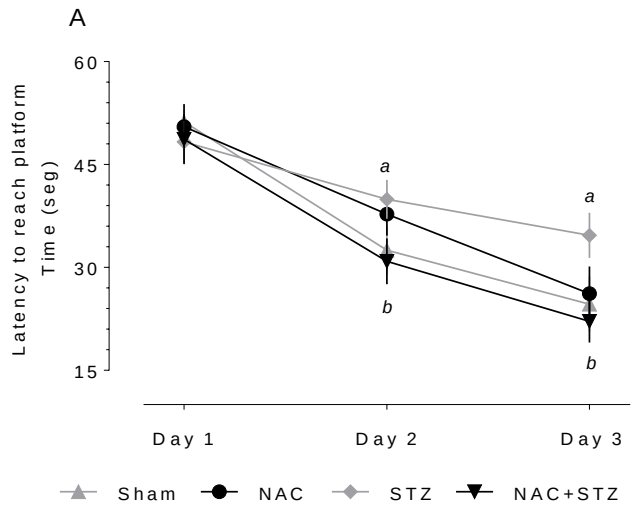
	OF (num)		SDPA (sec)	
	Crossings	**Rearings**	**Acquisition**	**Retention**
Sham	77.8 ± 9.8	42.0 ± 4.6	19.9 ± 3.5	209.3 ± 37.2
NAC	83.8 ± 9.2	45.8 ± 4.7	17.1 ± 5.9	150.8 ± 43.8
STZ	78.3 ± 7.7	40.6 ± 3.3	24.2 ± 3.6	92.2 ± 25.9[a]
NAC+STZ	84.8 ± 5.1	42.3 ± 3.8	18.5 ± 3.9	202.5 ± 22.9[b]

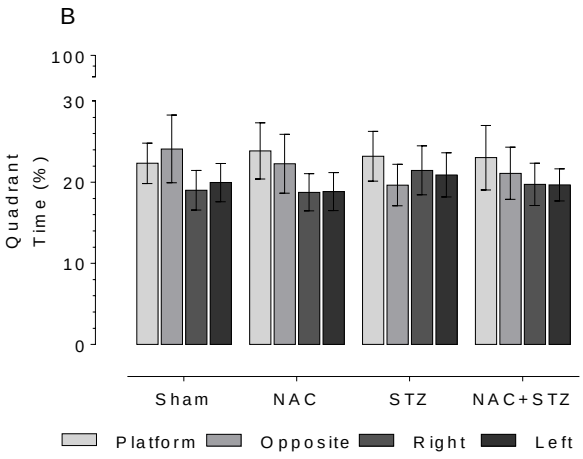
Data are reported as the mean ± S.E.M. for n = 8 animals per group. Statistical analysis was performed by two-way ANOVA followed by Duncan's test. [a] $p < 0.05$ as compared to the Sham group; [b] $p < 0.05$ as compared to the STZ group.

3.1.3. Morris water maze

Mice performance in the MWM task during the acquisition phase is presented in Fig. 1A. Along the 3 days of the acquisition phase, a decrease in time to find the platform was observed in all groups. Statistical analysis of latency time showed a significant STZ ($F_{1,28} = 5.497$; $p = 0.026$) main effect at day 2 and NAC ($F_{1,28} = 4.529$; $p = 0.042$) main effects at day 3. Post-hoc comparisons revealed that STZ increased the latency time to find the platform (~43% at day 2 and ~40% at day 3, respectively) when compared to Sham group. NAC treatment prevented this increase. Statistical analysis showed no

significant differences among groups in the time spends in each quadrant (Fig. 1B), and in the thigmotactic behavioral (Fig. 1C).





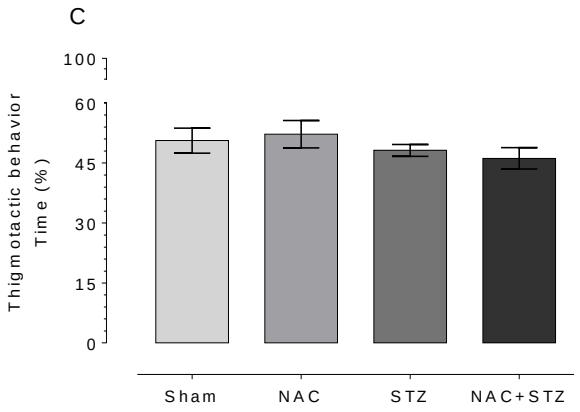
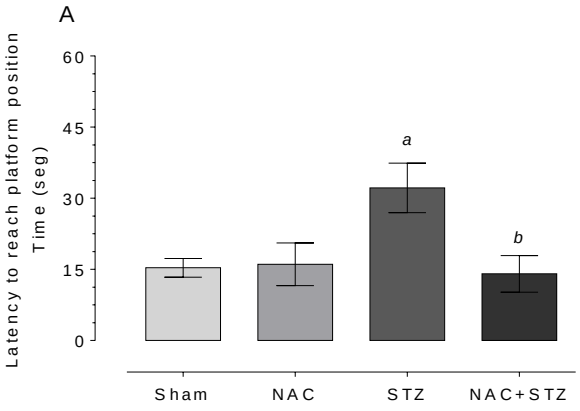


Fig. 1. Effects of NAC on STZ induced memory deficit in the Morris water maze task. (A) Latency (ies) to reach the platform in the acquisition phase, (B) time animals spent in each of the four quadrants of the pool during the acquisition phase and (C) thigmotactic behavior (time the animals spent swimming close to the wall zone) during the acquisition phase. Data represent the means ± S.E.M for n = 8 animals per group. Statistical analysis was performed by two-way ANOVA followed by Duncan's test. [a] $p < 0.05$ as compared to the Sham group; [b] $p < 0.05$ as compared to the STZ group.

Mice performance in the MWM task in the retention phase is presented in Fig. 2. Statistical analysis of latency time for the first crossing over the platform showed a significant NAC × STZ interaction ($F_{1,28} = 5.263$; $p = 0.030$) (Fig. 2A). Post-hoc comparisons demonstrated that STZ increased the time (~100%) when compared to Sham group and NAC treatment prevented this increase. Statistical analysis of the number of crosses over the platform local showed a significant NAC × STZ interaction ($F_{1,28} = 5.365$; $p = 0.028$) (Fig. 2B). Post-hoc comparisons revealed that STZ decreased (~45%) the number of crosses when compared to Sham group. The NAC treatment prevented this impairment.



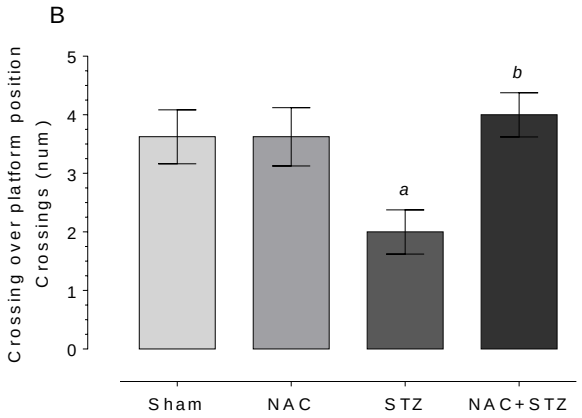


Fig. 2. Effects of NAC on STZ induced memory deficit in the Morris water maze. (A) latency (sec) to reach the platform place in the probe test and (B) number of crossing (num) over the former platform position. Data represent the means ± S.E.M for n = 8 animals per group. Statistical analysis was performed by two-way ANOVA followed by Duncan's test. [a] $p < 0.05$ as compared to the Sham group; [b] $p < 0.05$ as compared to the STZ group.

3.2. Biochemical assays

3.2.1. AChE and BChE activities

The AChE and BChE activities are presented in Fig. 3. Statistical analysis of brain cortex AChE activity showed a significant NAC × STZ interaction ($F_{1,28} = 5.675$; $p = 0.024$) (Fig. 3A). Post-hoc comparison demonstrated that STZ increased brain cortex AChE activity (~24%) when compared to the Sham group and NAC treatment prevented this increase. A significant NAC × STZ interaction ($F_{1,28} = 5.949$; $p = 0.022$) was also observed in brain cortex BChE activity. Post-hoc comparison showed a significant decrease (~51%) in enzyme activity in those animals that received NAC + STZ when compared to those of STZ group.

Statistical analysis of hippocampus AChE activity revealed a significant STZ main effect ($F_{1,28} = 5.079$; $p = 0.032$) (Fig. 3B). Post-hoc comparisons demonstrated that STZ increased enzyme activity (~52%) when compared to Sham group. NAC treatment prevented this increase. In hippocampus BChE activity a significant STZ main effect ($F_{1,28} = 8.269$; $p = 0.008$) was observed. Post-hoc comparisons demonstrated that STZ treatment increased the enzyme activity (~46%) when compared to Sham group. The NAC treatment prevented from this increase.

Striatum AChE and BChE activities were not affected by neither STZ nor NAC treatments (Fig. 3C).

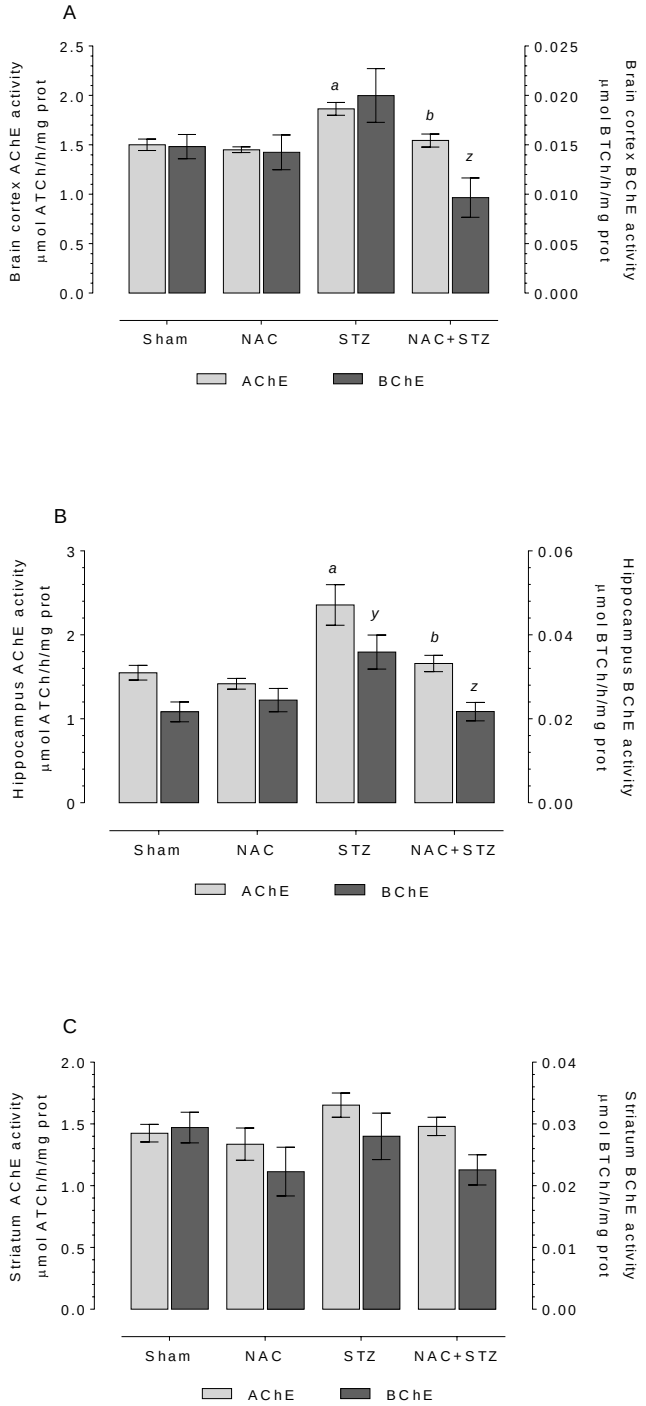


Fig. 3. Effect of NAC on AChE and BChE activity in the (A) brain cortex, (B) hippocampus and (C) striatum of mice that receive STZ injection. Data represent the means ± S.E.M for n = 8 animals per group. Statistical analysis was performed by two-way ANOVA followed by Duncan's test. ^{a} $p < 0.05$ as compared to the Sham

group for AChE activity; ^{b} $p < 0.05$ as compared to the STZ group for AChE activity. ^{y} $p < 0.05$ as compared to the Sham group for BChE activity. ^{z} $p < 0.05$ as compared to the STZ group for BChE activity.

3.2.2. ACh levels

The ACh levels are presented in Fig. 4. Statistical analysis of brain cortex ACh levels showed a significant NAC ($F_{1,28} = 7.281$; $p = 0.013$) and STZ ($F_{1,28} = 8.005$; $p = 0.009$) main effects. Post-hoc comparisons demonstrated that STZ decreased the neurotransmitter levels (~51%) when compared to Sham group and NAC treatment prevented this decrease. Analysis of hippocampus ACh levels revealed a significant NAC ($F_{1,28} = 6.161$; $p = 0.020$) and STZ ($F_{1,28} = 8.669$; $p = 0.007$) main effects. Post-hoc comparisons demonstrated that STZ decreased the neurotransmitter levels (~51%) when compared to Sham group. NAC treatment prevented from this decrease. A significant NAC ($F_{1,28} = 6.002$; $p = 0.022$) and STZ ($F_{1,28} = 8.748$; $p = 0.007$) main effects was also observed in striatum ACh levels. Post-hoc comparisons revealed that STZ decreased the neurotransmitter levels (~53%) when compared to Sham group.

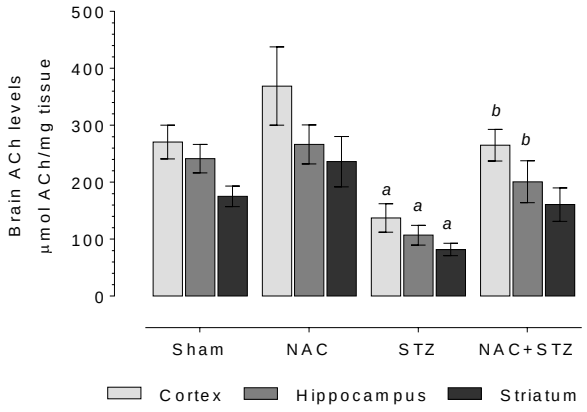


Fig. 4. Effect of NAC on brain ACh levels of mice that receive STZ injection. Data are reported as means ± S.E.M for n = 8 animals per group. Statistical analysis was performed by two-way ANOVA followed by Duncan's test. ^{a} $p < 0.05$ as compared to the Sham group; ^{b} $p < 0.05$ as compared to the STZ.

3.2.3. Energy-rich phosphate levels and glucose uptake capacity

The energy-rich phosphate levels and glucose uptake capacity are presented in Table 2. Statistical analysis of brain ATP and ADO levels showed a significant STZ main effect ($F_{1,12} = 6.535$; $p = 0.025$ and $F_{1,12} = 3.735$; $p = 0.003$, respectively). Post-hoc comparisons revealed that STZ decreased ATP level (~43%) and increased ADO level (~53%) when compared to sham group. Statistical analysis of hippocampus glucose

uptake showed a significant STZ main effect ($F_{1,12} = 5.644$; $p = 0.026$). Post-hoc comparisons revealed that STZ decreased hippocampus glucose uptake (~28%). The NAC treatment prevented the decrease induced by STZ.

Table 2. Effects of NAC administration on total brain energy-rich phosphate levels and brain glucose uptake in mice that received i.c.v. injection of STZ.

	Sham	NAC	STZ	NAC+STZ
	Energy rich phosphate (nmol/g tissue)			
ATP	133.4 ± 6.0	121.7 ± 32.0	76.0 ± 4.0[a]	86.1 ± 14.0[a]
ADP	600.1 ± 44.0	508.9 ± 95.0	472.1 ± 39.0	485.1 ± 70.0
AMP	2195.0 ± 127.0	1673.0 ± 383.0	1868.0 ± 191.0	2015.0 ± 137.0
ADO	2074.0 ± 195.0	1896.0 ± 365.0	3102.0 ± 327.0[a]	2638.0 ± 213.0[ab]
	Glucose uptake (µg/mg tissue)			
Cerebral cortex	144.0 ± 12.2	159.1 ± 10.3	127.1 ± 5.5	141.6 ± 9.8
Hippocampus	611.5 ± 29.5	626.6 ± 44.4	438.8 ± 43.1[a]	586.7 ± 63.9[b]
Striatum	489.4 ± 36.5	584.4 ± 81.9	531.6 ± 51.7	564.2 ± 44.3

Data are reported as means ± S.E.M for n = 4 animals per group. Statistical analysis was performed by two-way ANOVA followed by Duncan's test. [a] $p < 0.05$ as compared to the Sham group, [b] $p < 0.05$ as compared to the STZ group.

4. Discussion

In a previously report, we shown that NAC decreases mice brain AChE activity *in vitro* [36]. In the present study, we investigated the potentially neuroprotective effect of NAC on the cognitive deficit in mice treated with i.c.v. STZ. The results indicate that daily oral NAC administration at the dose of 50 mg/kg during nine consecutive days prevented from spatial and non-spatial learning and memory impairment in STZ-treated mice. Normalization of brain AChE and BChE activities, along with the increase in brain ACh concentration, seem to be the main causes of cognitive improvement due supplementation with NAC. The NAC treatment also prevented the energetic imbalance in brain of i.c.v. treated-STZ mice.

It is a fact that AD can be associated with motor impairments [37]. This fact can also interfere with the behavioral memory tests assessment. In this way, we care to assess motor activity of animals. The motor activity of the animals was not affected by any treatment as observed in the OP test performance. No effect of treatments was also observed in the acquisition phase in STDA. However, STZ affected the non-spatial long-term memory as shown by the decrease in the latency time to step down during the

retention phase. Furthermore, NAC *per se* did not alter the animal performance in this task. Nevertheless, NAC prevented the cognitive deficit in working memory.

The spatial learning and memory impairment [30] induced by the i.c.v. STZ in mice was possible to be observed in the MWM. The daily NAC administration prevented this impairment. This cognitive deficit was characterized by: (a) the increase in the latency to find the platform along the three days of the acquisition phase, (b) the increase in the latency to cross over the platform location during the probe test and (c) the reduction in the crossing number over the platform location during the probe test. Those animals that also received the NAC administration did not present those behaviors of cognitive deficit provoked by the i.c.v. STZ.

In the MWM, the procedural learning phase is dependent on the functional integrity of the striatum [38] while the spatial learning depends on the hippocampus [39], and both are modulated by the thalamic nuclei. Damages in those brain regions increase thigmotactic behavior and impair learning in MWM. Nevertheless, neither the animals that receive NAC nor STZ or both presented a significant thigmotactic behavior along the acquisition phase. Our results are in accordance with previous study where thigmotactism has no significant effect on a purely procedural of the task [40].

Over the last years, the use of AChE inhibitors and compounds that increase ACh synthesis has shown to be one of the most effective therapies in AD treating [41]. Nevertheless, agents that also target BChE may provide added benefits [42]. In healthy brain, BChE is responsible for approximately 20% of ACh hydrolysis. Nevertheless, brain BChE activity rises during AD [43] and increased BChE levels are associated with cortical and neocortical neuritic plaques development in AD [43,44]. In the present study i.c.v. STZ increased both AChE and BChE activities in cerebral cortex and hippocampus. The exact mechanism by which STZ increase brain ChEs activities is still unclear, and is the subject of our current research. On the other hand, oral NAC administration reversed the increase of both enzymes in both brain structures, as can be seen in those animals belonging to the NAC+STZ group, where enzymes activities are comparable to those of Sham group.

Along with the ChEs activities increase, a decrease in brain ACh concentration was also observed in those animals that receive the i.c.v. STZ. The NAC treatment prevented this decrease in cerebral cortex and hippocampus. The prevention of brain cortex and hippocampal ACh reduction facilitates the place learning [45] and the encoding of new information [46], respectively, as it was able to observe in both SDPA and MWM. It is important to emphasize that NAC here only prevented the decrease in

ACh levels induced by STZ and *per se* did not increase the neurotransmitter levels over those of Sham group, since high ACh levels might have beneficial or detrimental effects depending on the memory phase [46]. Unfortunately, the total recovery of striatum ACh levels was not achieved by NAC treatment. However, this fact seems not to have affected the mice memory acquisition in the behavioral tests. This result is in better agreement with the study of Kitabatake et al. [47] where alterations in the striatal cholinergic system showed no consequence on reference memory acquisition in the MWM. It is understandable that the decrease in the neurotransmitter level in those animals that received STZ may be consequence of increased ChEs activity. However, we do not rule out the hypothesis that this phenomenon may be due to a potential ChAT activity inhibition in those brain regions, notwithstanding, we did not perform this enzyme activity measure.

Impaired cerebral glucose utilization and energy metabolism represent very early abnormalities in initial stages of cognitive impairment [48]. A significant decrease in ATP and increase in ADO levels were observed in those animals that receive i.c.v. STZ, characterizing an inhibition on the synthesis of the energy-rich phosphates. Decrease ATP generation may affect energy-dependent central nervous system (CNS) neurotransmission processes and thus contributing to the decline in memory [49]. The i.c.v. STZ also decreased glucose uptake in hippocampus of mice, which could be related to the ATP levels decrease. NAC normalized glucose uptake in hippocampus and prevented the glucose utilization and ATP synthesis impairment in those animals that received STZ, fact that may be related to the improvement in the MWM. The NAC effects on brain glucose utilization and energy metabolism were previously reported by Martínes et al. [49], proving that NAC delays age-related memory impairment and age-related neurodegenerative diseases such as AD.

It is well documented that NAC displays its neuroprotective effect by different mechanism [50,51,52,53]. We here attribute the neuroprotective effect of NAC on its ability to prevent brain AChE and BChE increase. As mentioned before, NAC decreases mice brain AChE activity *in vitro* [36]. However, when dealing with an *in vivo* study, the NAC ability to cross the blood-brain barrier (BBB) is controversial. In one of the early studies on the subject, Sheffner et al. [54] reported that highest concentrations of NAC were seen in the brain of mice treated with S-NAC via oral. In 2003, Farr et al. [55] showed that NAC could completely cross the capillary wall to enter the mice brain and extracellular space at a rate of ~2.4 µL/g/min. It is possible that NAC ability to cross BBB may be dependent on the dose and administration via [56]. On the other hand, we are

aware that NAC also serves as a prodrug to the amino acid (AA) L-cysteine [57], which has similar NAC properties. This AA is transported into the brain by the AA transporters present in the BBB [58] mainly on the L-system [59]. Knowing this, we believe that NAC can cross the BBB and directly affect the ChEs activities. Nevertheless, we do not rule out the hypothesis that the beneficial effects on the cognitive impairment may be due to a NAC metabolite.

In summary, we here shown that i.c.v. STZ leads to a cognitive impairment in mice after nine days. Consequence of a brain energy metabolism disorder, which led to an increase of brain ChEs activities and reduction of the ACh levels. Oral NAC treatment prevents this impairment, mainly by diminishing the ChEs activities and restoring the ACh levels in the brain cortex and hippocampus. Thus, we conclude that mice exposed to STZ suffered cognitive impairment, affecting nonspatial long-term and espatial memory. However, NAC treatment prevented this disturbance, by restoring the brain energy metabolism and cholinergic system disorders.

We believe that these findings are of great relevance, given that NAC could modulate the cholinergic imbalance. However, the pharmacological manipulation of cholinergic activity might have beneficial or detrimental effects, which will depend on the suitability between the induced cholinergic activity and the memory phase [46]. In order to fully to elucidate the exact mechanisms by which NAC prevented the brain cholinergic system disturbance, additional studies are required.

Conflict of interest

All the authors here declare that there are no conflicts of interest.

Acknowledgements

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

References

[1] S, Coren, L.M. Ward, J.T. Enns, Sensation and Perception, 5th ed.: Harcourt Brace Collegem, New York, 1999.

[2] C.P. Ferri, M. Prince, C. Brayne, H. Brodaty, L. Fratiglioni, M. Ganguli, K. Hall, K. Hasegawa, H. Hendrie, Y. Huang, A. Jorm, C. Mathers, P.R. Menezes, E. Rimmer,

M. Scazufca. Global prevalence of dementia: a Delphi consensus study, Lancet. 366 (2005) 2112–2117.

[3] F. Giuliani, A. Vernay, G. Leuba, F. Schenk, Decrease behavioral impairments in an Alzheimer mice model by interfering with TNF-alpha metabolism, Brain. Research. Bulletin. 80 (2009) 302–308.

[4] M. Mancuso, V. Calsolaro, D. Orsucci, C. Carlesi, A. Choub, S. Piazza, G. Siciliano, Mitochondria, Cognitive Impairment, and Alzheimer's Disease, Int. J. Alzheimers. Dis. 6 (2009) pp: 951548.

[5] J.H. Xu, H.T. Hu, Y. Liu, Y.H. Qian, Z.H. Liu, Q.R. Tan, Z.J. Zhang, Neuroprotective effects of ebselen are associated with the regulation of Bcl-2 and Bax proteins in cultured mouse cortical neurons, Neurosci. Lett. 399 (2006) 210–214.

[6] L. Frölich, D. Blum-Degen, H.G. Bernstein, S. Engelsberger, J. Humrich, S. Laufer, D. Muschner, A. Thalheimer, A. Turk, S. Hoyer, R. Zochling, K.W. Boissl, K. Jellinger, P. Riederer, Brain insulin and insulin receptors in aging and sporadic Alzheimer's disease, J. Neural. Transm. 105 (1998) 423–438.

[7] W.L. Klein, ADDLs & protofibrils--the missing links?, Neurobiol. Aging. 23 (2002) 231–233.

[8] N. Sharma, R. Deshmukh, K.L. Bedi, SP600125, a competitive inhibitor of JNK attenuates streptozotocin induced neurocognitive deficit and oxidative stress in rats, Pharmacol. Biochem. Behav. 96 (2010) 386–394.

[9] H. Lannert, 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. 112 (1998) 1199–1208.

[10] B. Sharma, N. Singh, M. Singh, Modulation of celecoxib- and streptozotocin-induced experimental dementia of Alzheimer's disease by pitavastatin and donepezil, J. Psychopharmacol. 22 (2008) 162–171.

[11] S. Shoham, C. Bejar, E. Kovalev, D. Schorer-Apelbaum, M. Weinstock, Ladostigil prevents gliosis, oxidative-nitrative stress and memory deficit induced by intracerebroventricular injection of streptozotocin in rats, Neuropharmacol. 52 (2007) 836–843.

[12] J. Massoulie, L. Pezzementi, S. Bon, E. Krejci, F.M. Vallette, Molecular and cellular biology of cholinesterases, Progr. Neurobiol. 41 (1993) 31–91.

[13] M. Mesulam, C. Geula. Butyrylcholinesterase reactivity differentiates the amyloid plaques of aging from those of dementia. Annals. Neurol. 36 (1994) 722–727.

[14] M. Holden, C. Kelly, Use of cholinesterase inhibitors in dementia, Adv. Psychiatr. Treat. 8 (2002) 89–96.

[15] P. Taylor, Z. Radic, The cholinesterases: from genes to proteins. Ann. Rev. Pharmacol. Toxicol. 34 (1994) 281–320.

[16] M. Bazelyansky, E. Robey, J.F. Kirsch. Fractional diffusion limited component of reactions catalyzed by acetylcholinesterase. Biochem. 25 (1986) 125–30.

[17] G. Gron, I, Brandenburg, A.P. Wunderlich, M.W. Riepe, Inhibition of hippocampal function in mild cognitive impairment: targeting the cholinergic hypothesis. Neurobiol. Aging. 27 (2006) 78–87.

[18] R.A. Hut, E.A. Van der Zee, The cholinergic system, circadian rhythmicity, and time memory, Behav. Brain. Res. 221 (2011) 466–480.

[19] R.A. Sansone, L.A. Sansone, Chronic pain syndromes and borderline personality. Innov. Clin. Neurosci. 8 (2012) 10–14.

[20] M. Berk, G.S. Malhi, L.J. Gray, O.M. Dean, The promise of N-acetylcysteine in neuropsychiatry, Trends. Pharmacol. Sci. 34 (2013) 167–177.

[21] E. Azevedo, A.C. Mendes, M. Berk, E. Brietzke, To conduct the first systematic literature review of clinical trials of N-acetylcysteine (NAC) for the treatment of substance abuse disorders and addictive behaviors, Rev. Bras. Psiquiatr. 36 (1999) 168–175.

[22] S. Dodd, M. Maes, G. Anderson, O.M. Dean, S. Moylan, M. Berk, Putative neuroprotective agents in neuropsychiatric disorders, Prog. Neuropsychopharmacol. Bioll. Psychiatry. 42 (2013) 135–145.

[23] M. Haber, S.G. Abdel Baki, N.M. Grin'kina, R. Irizarry, A, Ershova, S. Orsi, R.J. Grill, P. Dash, P.J. Bergold, Minocycline plus N-acetylcysteine synergize to modulate inflammation and prevent cognitive and memory deficits in a rat model of mild traumatic brain injury, Exp. Neurol. 249 (2013) 169–177.

[24] A. Prakash, J.K. Kaira, A. Kumar, Neuroprotective effect of N-acetyl cysteine against streptozotocin-induced memory dysfunction and oxidative damage in rats, J. Basic. Clin. Physiol. Pharmacol. 26 (2015) 13–23.

[25] R. Agrawal, B. Mishra, E. Tyagi, C. Nath, R. Shukla, Effect of curcumin on brain insulin receptors and memory functions in STZ (ICV) induced dementia model of rat, Pharmacol. Res. 61 (2010) 247–252.

[26] T.J. Haley, W.G. McCormick, Pharmacological effects produced by intracerebral injection of drugs in the conscious mouse, Br. J. Pharmacol. Chemother. 12 (1957) 12–15.

[27] S.E. Laursen, J.K. Belknap, Intracerebroventricular injections in mice: some methodological refinements, J. Pharmacol. Methods. 16 (1986) 355–357.

[28] R.N. Walsh, R.A. Cummins, The open-field test: a critical review, Psychol. Bull. 83 (1976) 482–504.

[29] M. Sakaguchi, M. Koseki, M. Wakamatsu, E. Matsumura, Effects of systemic administration of casomorphin-5 on learning and memory in mice, Eur. J. Pharmacol. 530 (2006) 81–87.

[30] R. Morris, Developments of a water-maze procedure for studying spatial-learning in the rat, J. Neurosci. Methods. 11 (1984) 47–60.

[31] G.L. Ellman, K.D. Courtney, Jr.V. Andres, R.M. Featherstone, A new and rapid colorimetric determination of acetylcholinesterase activity, Biochem. Pharmacol. 7 (1961) 88–95.

[32] M.M. Bradford, A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principles of protein–dye binding, Anal. Biochem. 72 (1976) 248–254.

[33] S.B. He, G.W. Wu, H.H. Deng, A.L. Liu, X.H. Lin, X.H. Xia, W. Chen, Choline and acetylcholine detection based on peroxidase-like activity and protein antifouling property of platinum nanoparticles in bovine serum albumin scaffold, Biosens. Bioelectron. 62 (2014) 331–336.

[34] F. Özogul, A.K.D. Taylor, P. Quantick, Y. Özogul, A rapid HPLC-determination of ATP-related compounds and its applications to herring stored under modified atmosphere, Int. J. Food. Sci. Technol. 35 (2000) 549–554.

[35] A. Evans, M.E. Smith, Effect of beta-endorphin C-Terminal peptides on glucose uptake in isolated skeletal muscles of the mouse, Peptides. 18 (1997) 165–167.

[36] M. Costa, J. Bernardi, L. Costa, T. Fiuza, R. Brandão, M. Pereira. N-acetyl cysteine decease mice brain acetyl cholinesterase activity: An in vitro kinetic study, Enz. Eng. 5 (2015) 5–11.

[37] W.A. Mark, G.C. Gilmore, J. Kaye, C. Murphy, A. Wingfield, D.A. Bennett, A.L. Boxer, A.S. Buchman, K.J.Cruickshanks, D.P. Devanand, C.J. Duffy, C.M. Gall, G.A. Gates, A. Granholm, T. Hensh, R. Holzer, B.T. Hyman, F.R. Lin, A.C. McKee, J.C. Morris, R.C. Petersen, L.C. Silbert, R.G. Struble, J.Q. Trojanowski, J. Verghese, D.A. Wilson, S. Xu, L.I. Zhang. At the interface of sensory and motor dysfunctions and Alzheimer's disease, Alzheimers Dement. 11 (2015) 70-98

[38] R.J. McDonald, N.M. White, Parallel information processing in the water maze: evidence for independent memory systems involving dorsal striatum and hippocampus, Behav. Neural. Biol. 61 (1994) 260–270.

[39] R.G. Morris, E. Anderson, G.S. Lynch, M. Baudry, Selective impairment of learning and blockade of long-term potentiation by an N-methyl-D-aspartate receptor antagonist, AP5. Nature, 319 (1986) 774–776.

[40] D.P. Cain, F. Boon, M.E. Corcoran, Thalamic and hippocampal mechanisms in spatial navigation: a dissociation between brain mechanisms for learning how versus learning where to navigate, Behav. Brain. Res. 170 (2006) 241–256.

[41] S. Dodd, M. Maes, G. Anderson, O.M. Dean, S. Moylan, M. Berk, Putative neuroprotective agents in neuropsychiatric disorders, Prog. Neuropsychopharmacol. Biol. Psychiatry. 42 (2013) 135–145.

[42] C.G. Ballard, Advances in the Treatment of Alzheimer's Disease: Benefits of Dual Cholinesterase Inhibition. Eur. Neurol. 47 (2002) 64–70.

[43] E.K. Perry, B.E. Tomlinson, G. Blessed, K. Bergmann, P.H. Gibson, R.H. Perry. Correlation of cholinergic abnormalities with senile plaques and mental test scores in senile dementia. Br. Med. J. 2 (1978) 1457–1459.

[44] A. Guillozet, J.F. Smiley, D.C. Mash, M.M. Mesulam. Butyrylcholinesterase in the life cycle of amyloid plaques. Ann. Neurol. 42 (1997) 909–918.

[45] R.J. Mandel, F.H. Gage, L.J. Thal, Spatial learning in rats: correlation with cortical choline acetyltransferase and improvement with NGF following NBM damage, Exp. Neurol. 104 (1989) 208–217.

[46] J. Micheau, A. Marighetto. Acetylcholine and memory: A long, complex and chaotic but still living relationship, Behav. Brain Res. 221 (2011) 424–429.

[47] Y. Kitabatake, T. Hikida, D. Watanabe, I. Pastan, S. Nakanishi, Impairment of reward-related learning by cholinergic cell ablation in the striatum. Proc Natl. Acad. Sci. USA. 100 (2003) 7965–7970.

[48] Y.S. Jee, I.G. Ko, Y.H. Sung, J.W. Lee, Y.S. Kim, SE. Kim, B.K. Kim, J.H. Seo, M.S. Shin, H.H. Lee, H.J. Cho, C.J. Kim, Effects of treadmill exercise on memory and c-Fos expression in the hippocampus of the rats with Intracerebroventricular injection of streptozotocin, Neurosci. Lett. 443 (2008) 188–192.

[49] M. Martínez, A.I. Hernández, N. Martínez, N-Acetylcysteine delays age-associated memory impairment in mice: role in synaptic mitochondria, Brain. Research. 855 (2000) 100–106.

[50] F. Tchantchou, M. Graves, E. Rogers, D. Ortiz, T.B. Shea, N-acteyl cysteine alleviates oxidative damage to central nervous system of ApoE-deficient mice following folate and vitamin E-deficiency, J. Alzheimers. Dis. 7 (2005) 135–138.

[51] R. Stanislaus, A.G. Gilg, A.K. Singh, I. Singh, N-acetyl-L-cysteine ameliorates the inflammatory disease process in experimental autoimmune encephalomyelitis in Lewis rats, J. Autoimmune. Dis. 2 (2005) 2–4.

[52] T. Dawson, V. Dawson, Protection of the brain from ischemia, Cerebrovasc. Dis. 7 (1997) 349–352.

[53] S. Cuzzocrea, E. Mazzon, G. Costantino, I. Serraino, L. Dugo, G. Calabro, G. Cucionotta, A. De Sarro, A.P. Caputi, Beneficial effects of N-acetylcysteine on ischaemic brain injury, Br. J. Pharmacol. 130 (2000) 1219–1226.

[53] A.L. Sheffner, E.M. Medler, K.R. Bailey, D.G. Gallo, A.G. Mueller, H.P. Sarett, Metabolic studies with acetylcysteine. Biochem. Pharmacol. 15 (1996) 1523–1535.

[55] S.A. Farr, H.F. Poon, D. Dogrukol-Ak, J. Drake, W.A. Banks, E. Eyerman, D.A. Butterfield, J.E. Morley, The antioxidants a-lipoic acid and N-acetylcysteine reverse memory impairment and brain oxidative stress in aged SAMP8 mice. J. Neurochem. 84 (2003) 1173–1183.

[56] R.B. Shahripour, M.R. Harrigan, A.V. Alexandrov, N-acetylcysteine (NAC) in neurological disorders: mechanism of action and therapeutic opportunities. Brain Behav. 4 (2014) 108–122.

[57] M. Hacker, S.W. Messer II, K.A. Bachmann, Pharmacology: Principles and Practice. Academic Press, (2009). pp. 216–217.

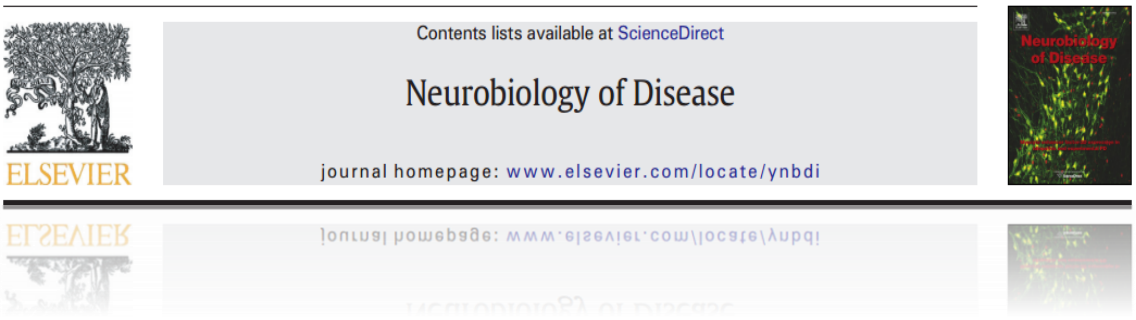
[58] H. Davson, M.B. Segal, Special aspects of the blood–brain barrier, in Physiology of the CSF and Blood–Brain Barriers, (1996) pp. 303–485. CRC Press, Boca Raton.

[59] L.A. Wade, H.M. Brady, Cysteine and cysteine transport at the blood-brain barrier. J. Neurochem. 37 (1981) 730–734.

3.3 Protocolo 3 (Manuscrito)

N-acetylcysteine treatment attenuates the cognitive impairment and synaptic plasticity loss induced by streptozotocin

Michael da Costa, Jamile Bernardi, Lidiane Costa, Tiago Fiuza, Ricardo Brandão, Maria Ribeiro, Joana Amaral, Cecília Maria Rodrigues e Maria Ester Pereira.



Neurobiology of Disease
YNBDI-S-16-00673

N-acetylcysteine treatment attenuates the cognitive impairment and synaptic plasticity loss induced by streptozotocin

Michael da Costa [1,3], Jamile Bernardi [1], Lidiane Costa [1], Tiago Fiuza [1], Ricardo Brandão [2], Maria F. Ribeiro [3], Joana D. Amaral [3], Cecília M.P. Rodrigues [3, †], Maria E. Pereira [1, ‡].

[1]Department of Biochemistry and Molecular Biology, Laboratory of Biochemistry and Toxicology, Center of Natural and Exact Sciences, Federal University of Santa Maria, UFSM Campus, Santa Maria, RS, Brazil

[2]Department of Pharmaceutical Sciences, Center of Health Sciences, Federal University of Pernambuco, UFPE Campus, Recife, PE, Brazil

[3]Research Institute for Medicines (iMed.ULisboa), Faculty of Pharmacy, Universidade de Lisboa, Lisbon, Portugal

† Corresponding author at: Research Institute for Medicines (iMed.ULisboa), Faculty of Pharmacy, Universidade de Lisboa, CP: 1649-003, Lisbon, Portugal. Tel.: +351-21-794-6490; Fax.: +351-21-794-6491. E-mail address: cmprodrigues@ff.ulisboa.pt (C.M.P. Rodrigues).

‡ Corresponding author at: Department Biochemistry and Molecular Biology, Center of Natural and Exact Sciences, Federal University of Santa Maria, Santa Maria, RS, Brazil. CEP: 97105-900, RS, Brazil. Tel: +55-55-3220-8799; Fax: +55-55-3220-8978. E-mail address: pereirame@yahoo.com.br (M.E. Pereira).

Abstract

Alzheimer's disease (AD) is a neurodegenerative disorder pathologically characterized by accumulation of extracellular amyloid-β (Aβ) peptide and intraneuronal hyperphosphorylated, leading to severe neuronal and glial structural changes and progressive cognitive decline. N-acetylcysteine (NAC) is a well-known pharmacological agent with pro-neurogenic properties and neuroprotective effects. In this study, we evaluated NAC protective effects on cognitive impairment and associated pathological markers in a streptozotocin (STZ)-induced sporadic dementia of AD type mice model. Animals were divided into six groups: I) Sham, II) NAC, III) physostigmine (PHY), IV) STZ, V) NAC+STZ and VI) PHY+NAC. NAC (5 mg/kg) and PHY (0.25 mg/kg) were administrated orally for 30 consecutive days and STZ (2.5 mg/kg) intracerebroventricularly at the first and third days. Novel object recognition (NOR, days 26-27) and Morris water maze (MWM, days 26-30) tasks were assessed to evaluate learning and memory. On the thirty-first day animals were euthanized and brains collected for biochemical analysis. Interestingly, our results showed that STZ treatment induced cognitive impairment in mice in the NOR and MWM tasks. Both NAC and PHY treatments prevented from this impairment. The increase in AChE activity and decrease in pTrkB and MnSOD levels caused by STZ in the cerebral cortex and hippocampus, were prevented by the NAC and PHY treatments. The decrease in SYN, MAP2 and GFAP expressions were also prevented by NAC and PHY treatments. In conclusion, NAC treatment prevented the cognitive impairment induced by STZ, normalizing the AChE activity and rescuing the synaptic plasticity loss. Our results suggest that NAC is a promising therapeutic strategy for the treatment of AD.

Key words: Acetylcholinesterase; memory impairment; synaptic plasticity.

Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by behavioral and psychological symptoms of dementia (Kristensen, 1990). This disease affects the memory and the abilities of judgment and reason, mainly by the loss of neurons and their synapses in the cerebral cortex and hippocampus (Resende et al., 2007). The cognitive impairment in AD is correlated with a significant reduction in the cholinergic activity (Francis et al. 1999). Despite the fact that AD has been mainly seen as a neuronal disease, a complex interaction between the different cell types in the brain exists. Therefore, it is conceivable that a pathological change in this interaction is responsible for promoting the cognitive decline since molecular and cellular features of astrocytes distinctly change during AD (Osborne et al., 2016). In fact, astrocytosis along with microglial activation are the mainly consequences of neuroinflammation, which is thought to play an important role in AD (Janssen et al., 2016).

Streptozotocin (STZ) is a naturally occurring chemical used in medicine for treating certain islets of Langerhans cancers (Brentjens & Saltz, 2001). Nevertheless, the intracerebroventricular (i.c.v.) STZ injection induces cognitive impairment affecting the cerebral cortex and hippocampus (Plaschke & Hoyer, 1993) being widely used as an animal model of sporadic AD (Tota et al., 2012). The cognitive dysfunction induced by STZ is due to a wide range of causes, including reduced cerebral energy metabolism (Lannert & Hoyer, 1998; Costa et al., 2016), neurodegeneration (Saxena et al., 2011) and cholinergic deficiency (Agrawal et al., 2009; Costa et al., 2016) among others. In this way, chemicals that decrease neurodegeneration and improve the cholinergic activity enhancement (Siddiqui & Levey, 1999; Holden & Kelly, 2002), like cholinesterase inhibitors (iChE), became the mainly options for the treatment of AD (Holden & Kelly, 2002).

N-acetylcysteine (NAC), a commonly used mucolytic and paracetamol antidote, is considered by the World Health Organization's one of the most important medication needed in a basic health system, and so it is present in the list as of essential medicines (WHO, 2014). The anti-inflammatory, anti-apoptotic and pro-neurogenic properties of NAC are known for almost two decades (Azevedo et al., 1999), and in recent years, its neuroprotective effects have been also described (Prakash et al., 2015). In this regard, the aim of this study was to determine the neuroprotective effect of chronical NAC administration in a mice model of STZ-induced cognitive impairment through the involvement of the cholinergic system, neurogenesis and astrogliosis.

2. Materials and methods

2.1. Animals

Experiments were conducted using male *Swiss* mice (~25 g) about two months old. Animals were maintained at 22 ± 2 °C with free access to water and food under a 12 h: 12 h light/dark cycle with lights on at 07:00. All manipulations were carried out between 08:00 and 16:00. Animals were used according to the guidance of the Committee on Care and Use of Experimental Animal Resources, the Federal University of Santa Maria, Brazil (Process Nº 088/2015) and the ARRIVE guidelines. All efforts were made to minimize animals' suffering and to reduce the number of animals used in the experiments.

2.2. Chemicals

NAC, physostigmine (PHY), STZ, acetylthiocholine (ATCh), 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), ethopropazine hydrochloride, β-actin, Triton X-100, ditiotreitol (DTT), Hoechst 33,258 and Fluoromount were acquired from Sigma-Aldrich Chemical Co. [St. Louis, MO, USA]. Anti-synaptophysin (anti-SYN), anti-microtubule associated protein 2 (anti-MAP2), anti-glial fibrillary acidic protein (anti-GFAP) were acquired from Millipore Corporation [Temecula, CA, USA]. Anti-ionized calcium binding adapter molecule 1 (anti-IBA1) was acquired from Wako Chemicals GmbH [Neuss, Germany]. Halt protease and phosphatase inhibitor cocktail were acquired from Thermo Fisher Scientific, Inc. [Rockford, IL, USA]. All other chemicals were obtained in an analytical grade or from standard commercial suppliers.

2.3. Experimental procedure

2.3.1. Memory impairment induction

Animals were divided into six groups (8 animals per group): I) Sham; II) NAC, III) PHY; IV) STZ; V) NAC+STZ and VI) PHY+STZ. For treatment one (T1), animals were treated for thirty consecutive days (p.o.) with a daily dose of distillated water (groups I and IV), NAC 5 mg/kg (groups II and V), or PHY 0.25 mg/kg (groups III and VI).

Physostigmine, which is a parasympathomimetic alkaloid and reversible iChE used in the clinical trials of Alzheimer's disease and prophylactic antidote to chemical warfare agent poisoning (Mach et al., 2004) was used as positive control. NAC and PHY

dose which showed no toxicity to animals were chosen based on a pilot study (data not shown).

For treatment two (T2), animals received (i.c.v., see below), at the first and third days, 5 µl/site of aCSF [aCSF (mmol/L): 147.0 NaCl; 2.9 KCl; 1.6 $MgCl_2$, 1.7 $CaCl_2$ and 2.2 $C_6H_{12}O_6$], pH = 7.4] (groups I, II and III) or STZ 2.5 mg/kg body weight dissolved in aCSF (groups IV, V and VI) (Agrawal et al., 2010). The i.c.v. administration of STZ (or aCSF) was performed by a "free hand" protocol with the bregma fissure as a reference point based on the previous published method (Haley & McCornick, 1957; Laursen & Belknap, 1986). Briefly, the animals were anesthetized with isoflurane and grasped firmly by the loose skin behind the head, the skin was pulled tight and a hypodermic needle (27 gauge) attached to a 25 µl Hamilton microsyringe was inserted perpendicularly through the skull into the brain. The site of injection was 2 mm either side of the brain midline on a line drawn through the anterior base of the ears; the depth of insertion of the needle was about 3 mm. To find out if the STZ was administered exactly into the cerebral ventricle, mice were killed by cervical dislocation and the brains were dissected and macroscopically examined.

2.4. Behavioural experiments

2.4.1. Open field test

Spontaneous locomotor activity was measured in the open field (OF) test (Walsh & Cummins, 1976) at the protocol day 25, previously to the memory task. The floor of the open field was divided into nine squares. Each animal was placed individually in the center of the arena, and the number of segments crossed (four-paw criterion) and rearings were recorded in a four min session.

2.4.2. Novel object recognition task

The novel object recognition task (NOR) was performed according to Bartolini et al. (1996) with some modifications. The day before to the experiment the mice were transferred to the laboratory, and allowed to explore and acclimate to the new environment for approximately 1h before the testing started. At the first day (D1) session (protocol day 26), each mouse was placed in the middle of the arena (white plastic box: 57 cm 35 cm 20 cm) and presented with two identical object (objects 1 and 2 – old objects) in two opposite corners, during 5 min. After that the animals were returned to their home cages. In the second day (D2) session (protocol day 27) the object 2 was

replaced for a new and not dissimilar object (object 3 – new object). Once again animal was placed in the middle of the arena and presented with two objects, the old familiar (object 1), and a new object (object 3) during five min, so the animal could explore both objects (recognition session). The objects were cleaned with 10% ethanol between each mouse and session. Object exploration was defined as mice sniffing or touching the object with its nose and/or forepaws. The time of exploration of each object (old and new) by each animal in the D2 was recorded. A discrimination index (DI) was calculated as a percentage of the time spent exploring each object over the total time spent exploring both objects.

2.4.3. Morris water maze test

Spatial learning and memory were assessed using the Morris water maze (MWM) task according to the method of Morris (1984). The maze consisted of a basin (diameter: 180 cm, wall height: 40 cm) made of black plastic and filled with water (25 ± 2 °C) at a height of 30 cm. The pool was placed in a room with several extra-maze visual cues. For five days (protocols days 26 to 30) the animals were submitted to four trials each day [starting in the north (N), then east (E), then south (S) and last west (W)]. The escape platform was hidden 0.5 cm below water level in the middle of the northwest (NW) quadrant. Once the mice have succeeded in find the platform it remained on this for at least 40 sec after each trial. Whenever mice failed to reach the escape platform within the 1 min cutoff period, they were guided to the platform and were left there for 40 sec. The escape latency to reach the platform was calculated as the mean of total time spent in four trials along each of the five days.

2.5. Biochemical analysis

2.5.1. Tissue collection

Twenty-four hours after the last behavioral test (protocol day 31), the animals were anesthetized with isoflurane and euthanized through cardiac puncture. Subsequently, under board certified personal guidance, the cerebral tissue was removed and the cerebral cortex and hippocampus were dissected.

2.5.1.1. Protein preparation and quantification

For the enzymatic activity and immunoblotting analyses, the dissected hippocampus and frontal cortex were homogenized in ice-cold Tris buffer (10 mmol/L

Tris-HCl, pH 7.6, 5 mmol/L $MgCl_2$, 1.5 mmol/L CH_3CO_2K, 1 % Nonidet P-40) supplemented with 2 mmol/L DTT and halt protease and phosphatase inhibitor cocktail in a 1:10 (w/v) ratio, for 30 min. Samples were then sonicated, the homogenate was centrifuged at 3,200 × g at 4 °C for 10 min, and the supernatant (S_1) fraction recovered. Protein concentrations were determined using the Bio-Rad protein assay kit, according to the manufacturer's recommendations.

2.5.2. Acetylcholinesterase activity

The acetylcholinesterase (AChE) activity was carried out according to the method of Ellman et al. (1961). The S_1 fraction was added into 0.1 mol/L potassium phosphate buffer (KPB), pH = 7.4, containing 0.02 mmol/L ethopropazine hydrochloride (to avoid butyrylcholinesterase (BChE) interference). Then, 0.3 mmol/L DTNB was added and the enzymatic reaction was initiated by addition of 0.45 mmol/L ATCh as substrate. Enzyme activity was spectrophotometrically measured at 412 nm for 2 min. Results are presented as percentage (%) change of Sham group.

2.5.3. Immunoblotting

Eighty micrograms of total protein extracts (S_1 fraction) were separated on 8 and 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After electrophoretic transfer onto nitrocellulose membranes and blocking with a 5 % milk solution, membranes were incubated overnight at 4 °C with the following primary antibodies: mouse monoclonal antibody reactive to phosphorylated tropomyosin receptor kinase B (pTrkB) and mouse monoclonal antibody reactive to manganese superoxide dismutase (MnSOD) (Santa Cruz Biotechnology Inc., Heidelberg, Germany). Finally, secondary goat anti-mouse or anti-rabbit IgG antibody conjugated with horseradish peroxidase (BioRad Laboratories, Hercules, CA, USA) was added for 2 h at room temperature. The membranes were processed for protein detection using the SuperSignal substrate (Pierce Biotechnology, Rockford, IL, USA). β-Actin (AC-15; Sigma-Aldrich) or GAPDH (32,233; Santa Cruz) were used as loading control. After rinsing with TBS/0.2% Tween 20, the immunoreactive proteins were visualized with Immobilon Western or SuperSignal West Femto substrate. GAPDH or β-actin was used as loading controls. Densitometric analyses were performed with the Image Lab software version 5.1 Beta (http://www.bio-rad.com/). Results are presented as percentage (%) change of Sham group.

2.5.4. Immunohistochemistry

Cerebral cortex and hippocampus were fixed in 4% paraformaldehyde for 48 h and stored in 30% sucrose and phosphate-buffered saline at 4 ºC. The treated tissues were further dehydrated and embedded in paraffin. Sequential coronal brain sections (4-mm thick) were obtained and mounted on SuperFrost-Plus glass slides. For immunostaining, brain structures were deparaffined and rehydrated, and antigen retrieval was performed by boiling the sections for 20 min in 10 mM citrate buffer, pH 6. The sections were then blocked for 1 h in Tris-buffered saline (TBS) containing 10% (v/v) normal donkey serum (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) and 0.1% (v/v) Triton X-100 and then incubated in appropriately diluted primary antibodies (1:100) overnight at 4 ºC. For synaptophysin (SYN) the presynaptic terminals were stained with a mouse monoclonal anti-SYN antibody; for microtubule associated protein 2 (MAP2) the neurons were stained with a rabbit polyclonal anti-MAP2 antibody; for glial fibrillary acidic protein (GFAP) the astrocytes were stained with a mouse monoclonal anti-GFAP antibody and for ionized calcium binding adapter molecule 1 (IBA1) the microglia were stained with a rabbit polyclonal anti-IBA1 antibody. After washing with TBS/0.025% Tween20, the primary antibodies were developed with diluted (1:100) Alexa Fluor 568 (anti-mouse) or Alexa Fluor 594 (anti-rabbit) conjugated secondary antibodies for 2 hours at room temperature. After rinsing, the sections were counterstained with Hoechst 33,258 and mounted on Fluoromount.

2.5.4.1. Image analysis

All images were captured using an Axioskop fluorescence microscope (Carl Zeiss GmbH, Hamburg, Germany). At least three images per cortical and hippocampal regions were acquired for each animal and converted to gray-scale with an 8-bit format. Semiquantitative analysis of SYN, MAP2, GFAP, and IBA1 were performed with ImageJ version 1.46r software (https://imagej.nih.gov/ij/). The background of each set of images was subtracted, and a threshold optical density was determined and held constant. Mean gray values obtained for SYN, MAP2, GFAP, and IBA1 immunostaining in the six groups were normalized to the mean gray values units of Sham group and are presented as percentage (%) change of Sham group.

2.6. Data presentation and statistical analysis

All experiments results are given as the mean (s) ± S.E.M. Statistical analyses were performed using one-way ANOVA followed by Tukey's multiple comparisons test when appropriated. Values of $p < 0.05$ were considered statistically significant. Calculations

were performed using software GraphPad Prism 6.01. La Jolla, CA – USA (https://www.graphpad.com).

3. Results

3.1. Behavioural tests

3.1.1. Open field test (OF)

Mice performances in the OF test are presented in Table 1. No effect on spontaneous nor exploratory activity was observed by any of the treatments.

Table 1. Spontaneous locomotor and exploratory activity in the open field-test of mice that received i.c.v. injection of STZ.

	Open field (num)	
	Crossings	**Rearings**
Sham	66.88 ± 5.47	20.75 ± 2.29
Nac	62.75 ± 5.29	19.38 ± 3.01
Phy	65.13 ± 5.48	20.25 ± 2.69
Stz	64.00 ± 4.01	16.50 ± 1.71
Nac+Stz	56.88 ± 5.79	18.13 ± 2.51
Phy+Stz	63.88 ± 5.55	17.63 ± 3.04

Data are reported as the mean ± S.E.M. for n = 6 to 8 animals per group.

3.1.2. Novel object recognition task (NOR)

Mice performances in the NOR are presented in Fig. 1. Statistical analysis yielded a significant difference among the groups ($F_{5,42} = 3.505$, $p = 0.011$). Post-hoc comparisons revealed that STZ decreased (~23%) the time that the animals explore the new object when compared to the Sham group. Both NAC and PHY treatments prevented this effect.

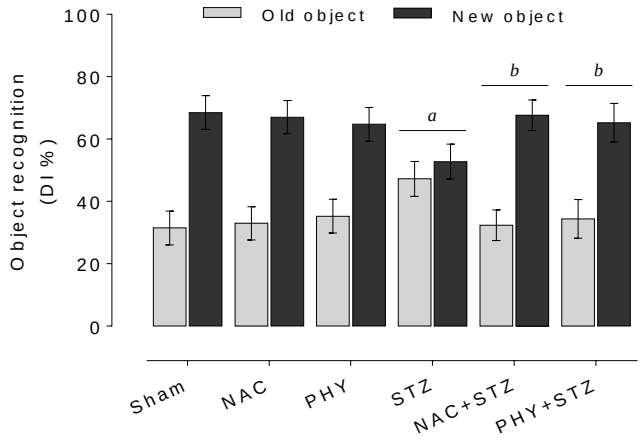


Fig. 1. Mice performance in the object recognition task. Data represent the means ± S.E.M for n = 6 to 8 animals per group. Statistical analysis was performed by one-way ANOVA followed by Tukey's test. [a] $p < 0.05$ as compared to the Sham; [b] $p < 0.05$ as compared to the STZ.

3.1.3. Morris water maze test (MWM)

Mice performances in the MWM are presented in Fig. 2. Along the five days of test, a decrease in time to find the hidden platform was observed in all groups. Statistical analysis showed a significant difference among the groups ($F_{5,42} = 8.792, p < 0.001$). Post-hoc comparisons showed that STZ increased (~12%) the latency to reach the platform starting at the second day when compared to the Sham group. This increase continued along the remaining four days, (being ~72% at the fifth day). The PHY and NAC treatments prevented this increase starting at the second and third days, respectively.

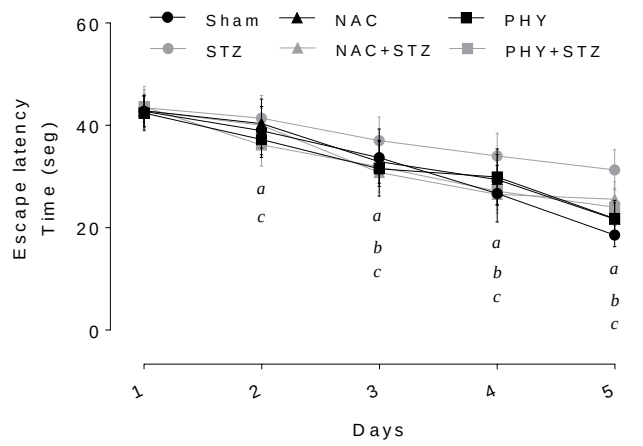


Fig. 2. Escape latency (ies) to reach the platform in the Morris water maze task. Data represent the means ± S.E.M for n = 6 to 8 animals per group. Statistical analysis was performed by one-way ANOVA followed

by Tukey's test. [a] $p < 0.05$ as STZ compared to the Sham; [b] $p < 0.05$ as NAC+STZ compared to the STZ; [c] $p < 0.05$ as PHY+STZ compared to the STZ.

3.2. Biochemical analysis

3.2.1. Acetylcholinesterase activity

The AChE activities are presented in Fig. 3. Statistical analysis of the cerebral cortex AChE activity showed a significant difference among the groups ($F_{5,30} = 7.380$, $p < 0.001$). Post-hoc comparisons revealed that STZ increased (~10%) the cerebral cortex AChE activity when compared to the Sham group. The NAC and PHY treatments prevented from this increase. Statistical analysis of the hippocampus AChE activity showed a significant difference among the groups ($F_{5,30} = 9.203$, $p < 0.001$). Post-hoc comparisons demonstrated that STZ treatment increased (~16%) the hippocampus AChE activity when compared to the Sham group. Both NAC and PHY treatments prevented from this increase.

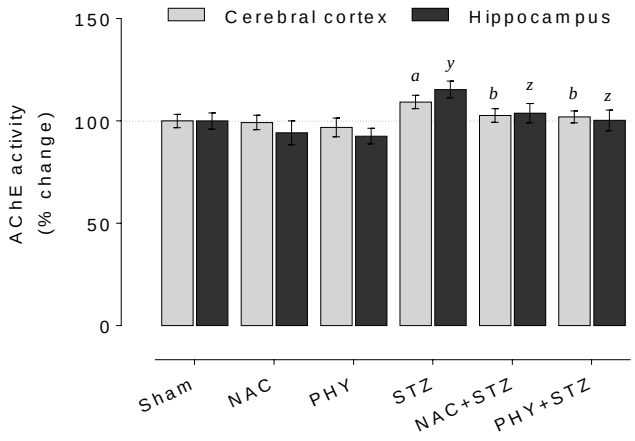


Fig. 3. Mice AChE activity. Data represent the means ± S.E.M for n = 6 to 8 animals per group. Statistical analysis was performed by one-way ANOVA followed by Tukey's test. [a] $p < 0.05$ as compared to the Sham; [b] $p < 0.05$ as compared to the STZ (for cerebral cortex). [y] $p < 0.05$ as compared to the Sham; [z] $p < 0.05$ as compared to the STZ (for hippocampus).

3.2.2. Immunoblotting

The pTrkB levels are presented in Fig. 4. Statistical analysis of the cerebral cortex pTrkB levels showed a significant difference among the groups ($F_{5,30} = 61.16$, $p < 0.001$). Post-hoc comparisons revealed that STZ decreased (~40%) the cerebral cortex pTrkB levels when compared to the Sham group (Fig. 4A). The NAC and PHY treatments

prevented from this decrease. An increase (~18%) was observed also in those animals from NAC group. Statistical analysis of the hippocampus pTrkB levels showed a significant difference among the groups ($F_{5,30} = 12.07$, $p < 0.001$). Post-hoc comparisons demonstrated that STZ treatment decreased (~20%) the hippocampus pTrkB levels when compared to the Sham group (Fig. 4B). The NAC and PHY treatments also prevented from this decrease.

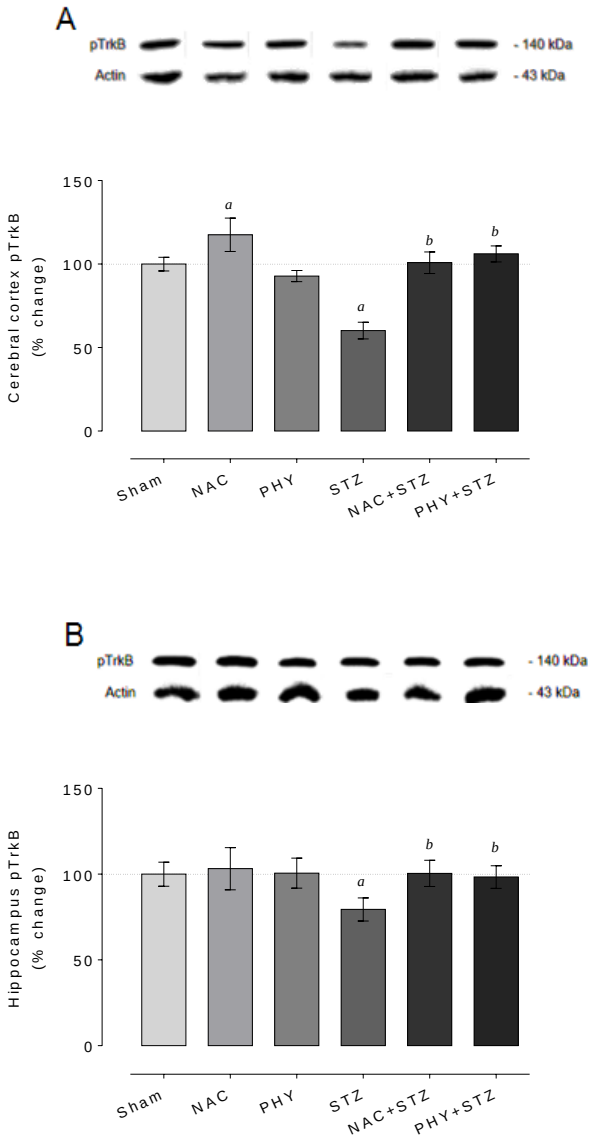


Fig. 4. Mice pTrkB levels in (A) cerebral cortex and (B) hippocampus. Data represent the means ± S.E.M for n = 6 to 8 animals per group. Statistical analysis was performed by one-way ANOVA followed by Tukey's test. [a] $p < 0.05$ as compared to the Sham; [b] $p < 0.05$ as compared to the STZ.

The MnSOD levels in cerebral cortex and hippocampus are presented in Fig. 5. Statistical analysis of the cerebral cortex MnSOD levels showed a significant difference among the groups ($F_{5,30} = 13.95$, $p < 0.001$). Post-hoc comparisons revealed that STZ decreased (~28%) MnSOD level in cerebral cortex when compared to the Sham group (Fig. 5A). Both NAC and PHY treatments prevented from this decrease. No effect of neither treatments was observed in hippocampus MnSOD levels (Fig. 5B).

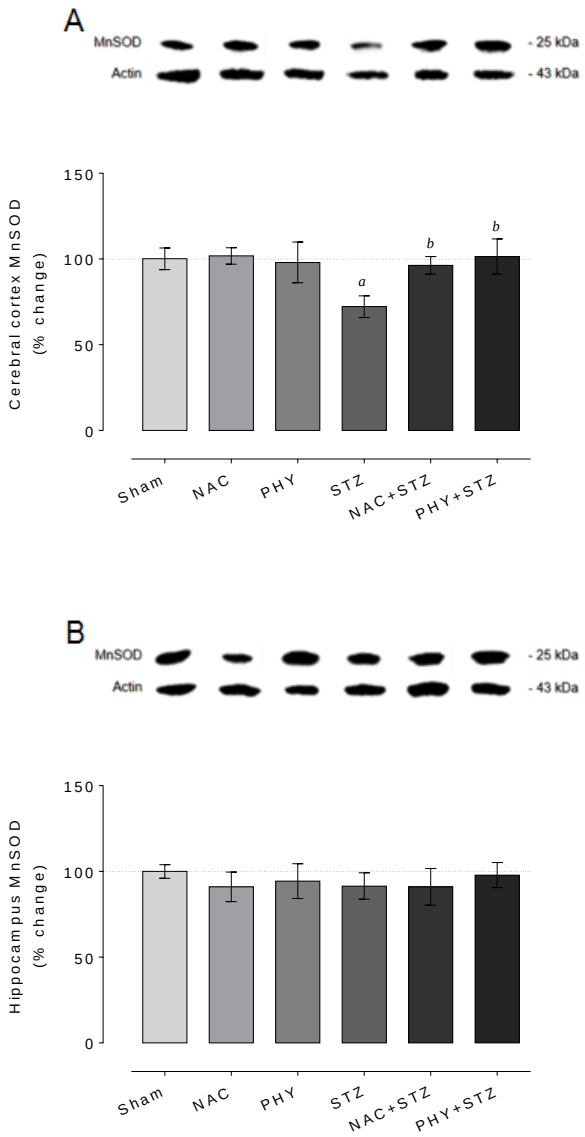


Fig. 5. Mice MnSOD levels in (A) cerebral cortex and (B) hippocampus. Data represent the means ± S.E.M for n = 6 to 8 animals per group. Statistical analysis was performed by one-way ANOVA followed by Tukey's test. [a] $p < 0.05$ as compared to the Sham; [b] $p < 0.05$ as compared to the STZ.

3.2.3. Immunohistochemistry

The SYN expression in cerebral cortex and hippocampus are presented in Fig. 6. Statistical analysis of the cerebral cortex SYN revealed a significant difference among the groups ($F_{5,30} = 9.465$, $p < 0.001$). Post-hoc comparisons showed that STZ decreased (~27%) SYN when compared to the Sham group (Fig. 6A). The NAC treatment prevented this decrease in cerebral cortex SYN. Statistical analysis of the hippocampus SYN expression revealed a significant difference among the groups ($F_{5,30} = 12.07$, $p < 0.001$). Post-hoc comparisons show that STZ decreased (~27%) SYN when compared to the Sham group (Fig. 6B). Both NAC and PHY treatments were effective in preventing this decrease.

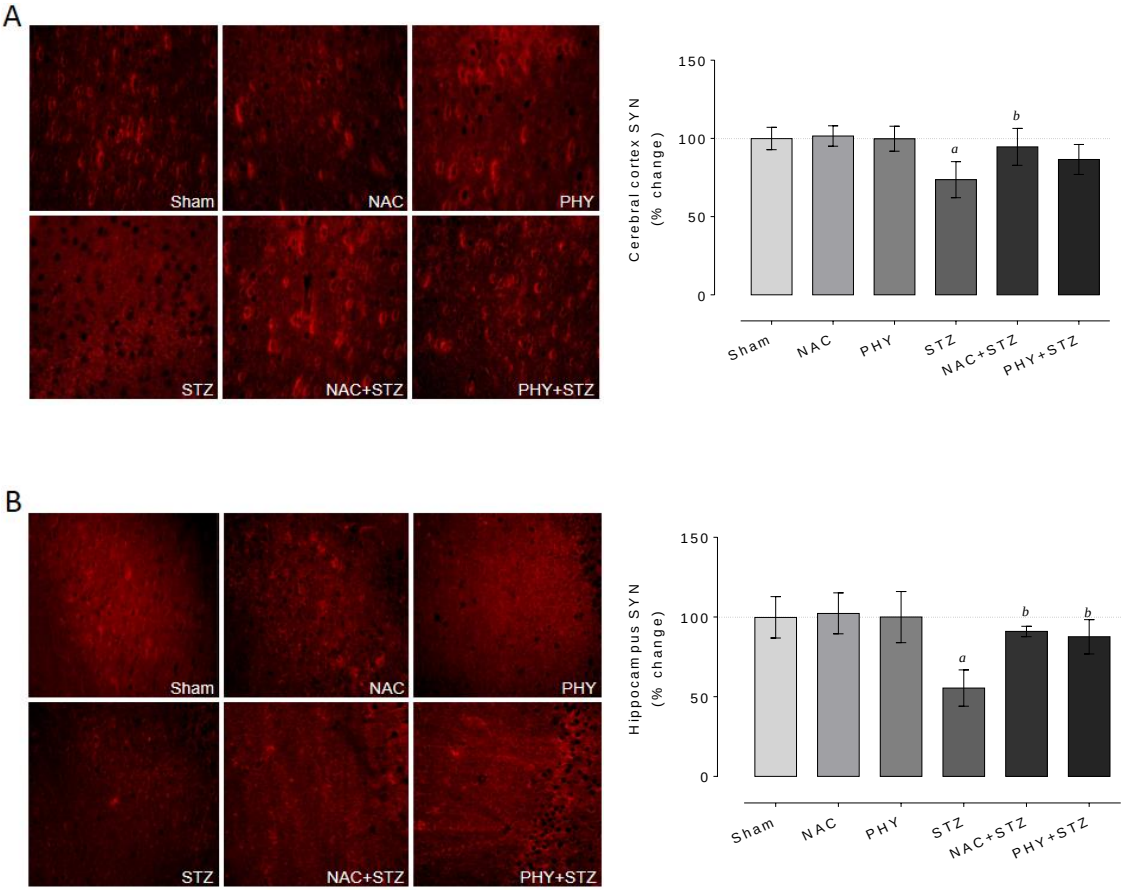


Fig. 6. Mice SYN expression in (A) cerebral cortex and (B) hippocampus. Data represent the means ± S.E.M for n = 6 to 8 animals per group. Statistical analysis was performed by one-way ANOVA followed by Tukey's test. [a] $p < 0.05$ as compared to the Sham; [b] $p < 0.05$ as compared to the STZ.

The MAP2 expression in cerebral cortex and hippocampus are presented in Fig. 7. Statistical analysis of cerebral cortex MAP2 revealed a significant difference among the groups ($F_{5,30} = 10.07$, $p < 0.001$). Post-hoc comparisons showed that STZ decreased

(~22%) the MAP2 when compared to the Sham group (Fig. 7A). The NAC and PHY prevented from this decrease in cerebral cortex MAP2. Statistical analysis of hippocampus MAP2 expression revealed a significant difference among the groups ($F_{5,30} = 8.122$, $p < 0.001$). Post-hoc comparisons yielded that STZ decreased (~30%) the MAP2 when compared to the Sham group (Fig. 7B). Once again, both NAC and PHY treatments prevented from this decrease.

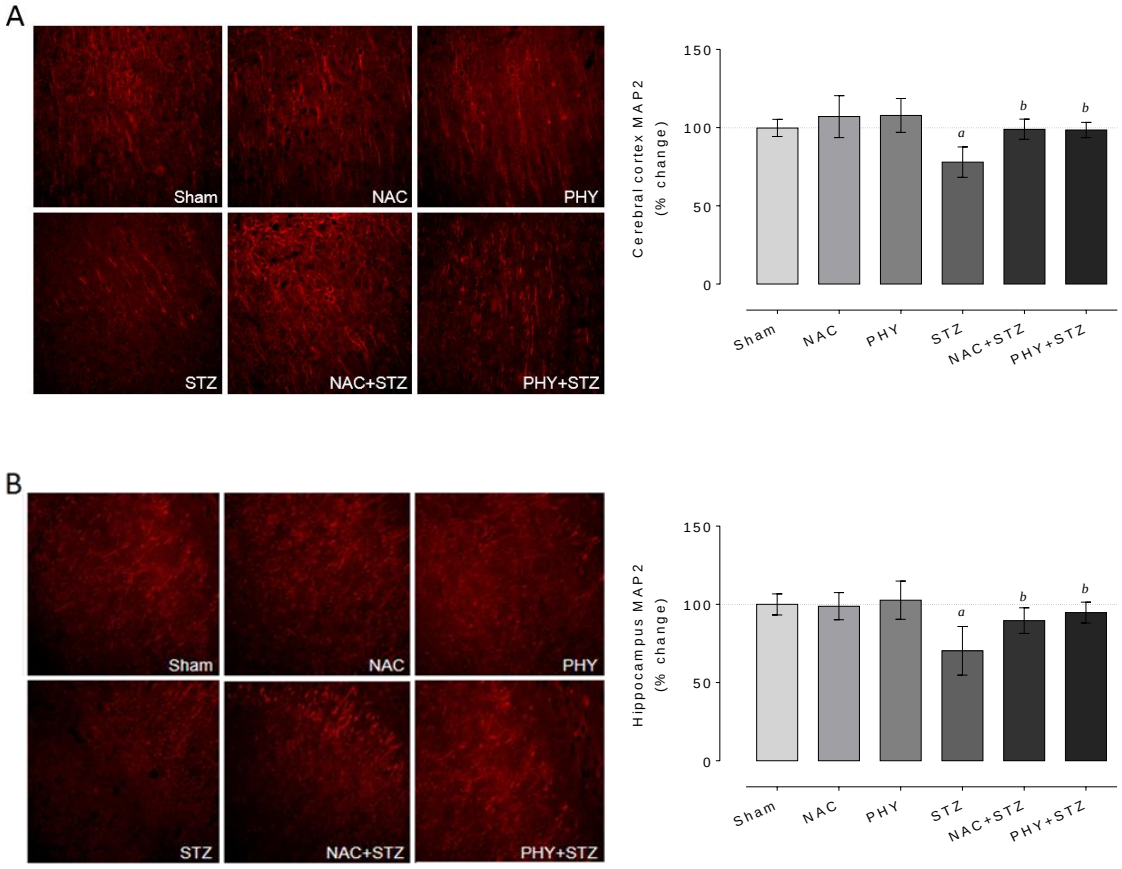


Fig. 7. Mice MAP2 expression in (A) cerebral cortex and (B) hippocampus. Data represent the means ± S.E.M for n = 6 to 8 animals per group. Statistical analysis was performed by one-way ANOVA followed by Tukey's test. [a] $p < 0.05$ as compared to the Sham; [b] $p < 0.05$ as compared to the STZ.

The GFAP expression in cerebral cortex and hippocampus are presented in Fig. 8. Statistical analysis of cerebral cortex GFAP revealed a significant difference among the groups ($F_{5,30} = 5.326$, $p = 0.001$). Post-hoc comparisons showed that STZ decreased (~24%) the GFAP when compared to the Sham group (Fig. 8A). Nevertheless, NAC and PHY treatments prevented from this decrease. Statistical analysis of hippocampus GFAP expression revealed a significant difference among the groups ($F_{5,30} = 20.09$, $p <$

0.001). Post-hoc comparisons showed that STZ decreased (~28%) the GFAP when compared to the Sham group (Fig. 8B). Both NAC and PHY treatments partially prevented this decrease.

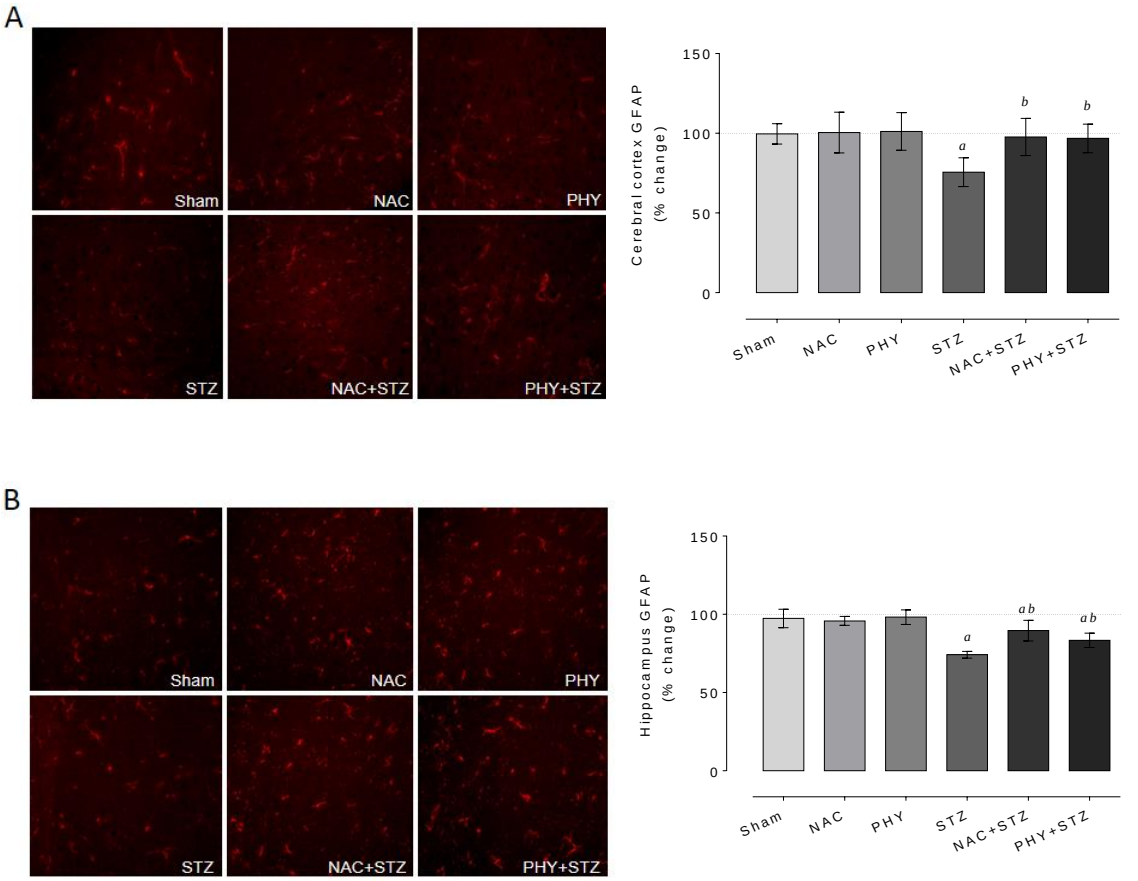


Fig. 8. Mice GFAP expression in (A) cerebral cortex and (B) hippocampus. Data represent the means ± S.E.M for n = 6 to 8 animals per group. Statistical analysis was performed by one-way ANOVA followed by Tukey's test. [a] $p < 0.05$ as compared to the Sham; [b] $p < 0.05$ as compared to the STZ.

The IBA1 expression in cerebral cortex and hippocampus are presented in Fig. 9. Statistical analysis of IBA1 showed no significant differences among the groups ($p > 0.05$) neither in cerebral cortex (Fig. 9A) nor hippocampus (Fig. 9B).

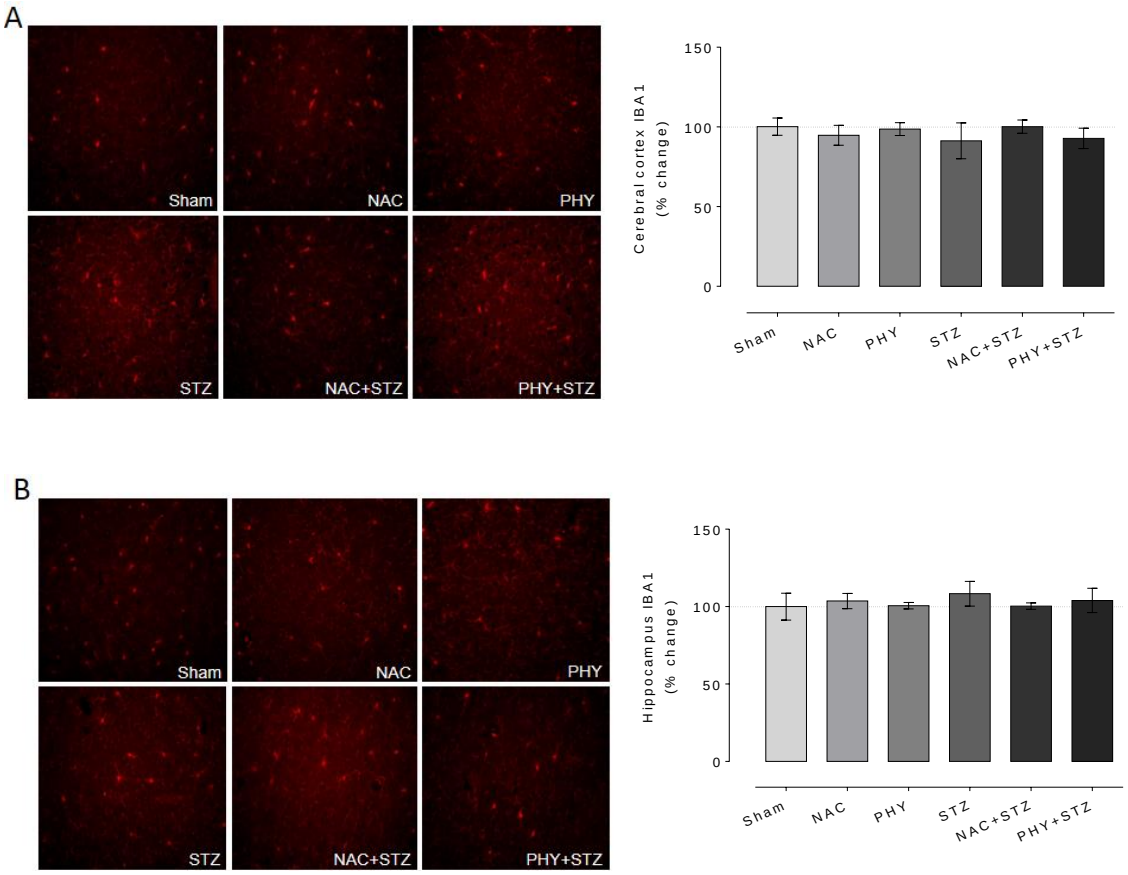


Fig. 9. Mice IBA1 expression in (A) cerebral cortex and (B) hippocampus. Data represent the means ± S.E.M for n = 6 to 8 animals per group. Statistical analysis was performed by one-way ANOVA followed by Tukey's test.

4. Discussion

Recently we showed the neuroprotective effect of NAC against the cognitive impairment induced by STZ in mice in a short period (Costa et al., 2016). Here, we aimed to investigate the neuroprotective effects of therapeutic NAC chronical administration on the cognitive impairment induced by STZ in mice. The daily 5 mg/kg NAC oral administration for 30 consecutive days prevented the memory impairment in STZ-treated mice. This memory impairment was characterized by an increase in AChE activity, and a decrease in pTrkB levels and SYN expression in cerebral cortex and hippocampus, which we believe were the main causes of this disturbance. NAC treatment prevented from the disturbance induced by STZ.

As mentioned before, the i.c.v. STZ injection induces cognitive impairment (Plaschke & Hoyer, 1993) and is considered an animal model of sporadic AD (Tota et al., 2012). In the AD pathology the sensory and motor regions of the central nervous

system (CNS) are affected (Mark et al., 2015). Here, no impairment in the exploratory behavior and locomotor activity was established by STZ treatment as observed in the OF task. Thus, we can also rule out the hypothesis of locomotor activity interference in behavioral tests.

The STZ led to a cognitive impairment in mice as observed in the NOR task. This task depends mainly on the curiosity of the rodents and preference for novelty (Robbins, 1977) and evaluates the non-spatial recognition memory. Those animals that received the i.c.v. STZ injection explored both (old and new) objects for a similar period on the second day. And therefore, they explored the new object for a shorter period when compared to the animals in the Sham group. The non-spatial recognition memory impairment induced by STZ was prevented by both NAC and PHY treatments, once the animals belonging to groups NAC+STZ and PHY+STZ showed similar exploration of the new object levels compared to the Sham group. Along with this, no differences in either locomotor activity or general exploratory activity were observed during the D1 and D2 among the different experimental groups.

The non-spatial memory tasks often involve stimuli that are relatively unfamiliar or completely novel, on the other hand, the spatial memory task typically tax the animal ability to discriminate between two or more familiar environments (Pitsikas and Markou, 2014). An impairment in the spatial learning and memory was found in those animals that received the i.c.v. STZ injection as observed in the MWM task. Those phenomena were characterized by the increase in the latency to find the platform along the five days of the test. This increase can indicate cortical (D'Hooge and De Deyn, 2001) and hippocampal (Morris et al., 1982) damage in those animals. The NAC and PHY treatments prevented this impairment caused by the STZ. This prevention was observable starting from the second and third trials, for PHY and NAC, respectively. No difference in locomotor activity were also observed. This could be seen from the first day of test, where the animals showed similar latencies to find the platform. Strengthening the hypothesis that the increase in latency to find the platform was due to the STZ effect.

Acetylcholine (ACh) plays a critical role in cognitive functions, mainly on learning and memory (Klinkenberg et al., 2011). In AD a marked ACh hypofunction is observed (Figueró et al., 2010), usually result of decreased choline acetyltransferase (ChAT) (Lane et al., 2004) and increased AChE levels (de la Monte and Wands, 2008). Here, the STZ led to an increase in cerebral cortex and hippocampus AChE activity, which could result in increased ACh degradation. Our results are in better accordance with previously studies of Pinton et al. (2013) and Costa et al. (2016), where the i.c.v. STZ injection

increased AChE activity those brain areas. The exact mechanism by which STZ increases brain AChE is not entirely clear. Notwithstanding, this phenomenon maybe due to an increased expression of genes encoding AChE (Lester-Collet al., 2006). The NAC, as well the iChE PHY treatments prevented this increase in the cerebral cortex and hippocampus. This normalization in AChE activity can be related with the cognitive impairment prevention observed in the MWM and NOR tests since cerebral cortex ACh is important for attention and (object) recognition, while hippocampus ACh is important for acquisition of new information (Klinkenberg et al., 2011).

The tropomyosin receptor kinase B (TrkB) is a receptor for the Brain Derived Neurotrophic Factor (BDNF) (Zunino et al., 2016). The receptor phosphorylated form (pTrkB) is formatted after the specific association with the BDNF. Decreased levels of pTrkB were observed in cerebral cortex and hippocampus in those animals of STZ group, reflecting low levels of BDNF in those brain areas. BDNF plays an important role in neuronal growth, cell differentiation, neuronal survival, synaptogenesis and synaptic plasticity (Acheson et al., 1994; Huang and Reichardt, 2001). Furthermore, BDNF participates in learning and memory processes (Yamada and Nabeshima, 2003; Bekinschtein, 2008), been very important for long-term memory (Bekinschtein, 2008; Lu et al., 2008). BDNF is initially synthesized in the endoplasmic reticule as preproBDNF and finally converted in mature (BDNF) in the trans-Golgi by endoproteases (Mowla et al., 1999). Here, the pTrkB levels decrease may be reflection of reduced BDNF synthesis, consequence of disturbed energy metabolism. A decrease in mitochondrial MnSOD levels in cerebral cortex were also observed in those animals that received the i.c.v. STZ, which could lead in to mitochondrial dysfunction (Konzack et al., 2015) and decreased adenosine triphosphate (ATP) synthesis. Both, NAC and PHY treatments prevented from this decrease in pTrkB and MnSOD levels. The NAC prevention of brain energy metabolism disruption induced by STZ was already demonstrated in a previously work from our laboratory (Costa et al., 2016).

Synapses are the principal unit of intracellular communication in neuronal circuits (Sze et al., 1997) and its quantification can be made by the use of SYN immunostaining (Calhoun et al.,1996). The loss of synapses are hallmarks of AD (Tampellini et al., 2010). Here, those animals belonging to the STZ group showed decreased expression of SYN and therefore loss of synaptic plasticity in cerebral cortex and hippocampus. This synaptic function is important for memory and learning in animal models (Bliss and Lomo, 1973), which was evidenced here by the poor performance of those animals in the NOR and MWM test. The NAC treatment prevented this decrease in cerebral cortex and

hippocampus. The cerebral cortex SYN expression decrease was also prevented by the PHY treatment. The maintenance of the neuronal processes in the CNS is dependent upon the state of assembly of microtubules (Ladrech et al., 2003) and the protein that maintain the dendritic structure through interaction with microtubules is the MAP2 (Shelton et al., 2014). Decreased expression of cerebral cortex and hippocampus MAP2 were also observed in those animals that receive i.c.v. STZ. This protein is an important regulator of neurotic development and is regulated by development and experience-dependent plasticity (Shelton et al., 2014). This decrease in MAP2 expression correlate with the increased latency to reach the platform exhibit by those animals in the MWM test. Nevertheless, those animals that were also treated with NAC and PHY did not present decreased expression of MAP2, neither in cerebral cortex nor hippocampus. Also showing an improvement in the development plasticity, regarding the environmental interaction and learning information during the test sections observable in the MWM test.

The astrocytes are involved in various forms of dementia, including AD (Verkhratsky et al., 2010) and the intermediate filaments of astrocytes are composed mainly by GFAP (Sofroniew and Vinters, 2010). Decreases in the expression of GFAP are invariably associated with detrimental conditions in the CNS (Pekny and Pekna, 2004). Here, the STZ led to a decrease in cerebral cortex and hippocampus GFAP expression in mice. Significant decrease in GFAP expression had already been reported in the brain of very old AD patients (Verkhratsky and Butt, 2013) and as consequence of neurodegeneration (Johnston-Wilson et al., 2000). This could be consequence of astroglial atrophy, which appears at the very early stages of neurodegenerative diseases (Verkhratsky et al., 2010). The NAC and PHY treatments prevented the decrease in GFAP expression in cerebral cortex. This prevention by NAC and PHY was partly in the hippocampus. Along with the astrocyte, another important glia responsible for the CNS homeostasis is the microglia. The expression of IBA1, a calcium-binding protein which expression is associated with microglia activation (Ito et al., 2001) was not affected by the STZ, neither in cerebral cortex nor hippocampus. Thus, indicating the absence of neuroinflammation, which is a neuropathological hallmark of the AD (Janssen et al., 2016).

In summary, the i.c.v. STZ lead to a cognitive impairment in mice after thirty days. The NAC treatment protects from this impairment. We believe that this protective effect of NAC could be due the normalization of the AChE activity increase induced by STZ. This increase in AChE expression and activity may be consequence of amyloid deposition in neurons and astrocytes (Sáez-Valero et al., 2003), and can promote

neurodegeneration, cell death (Talesa, 2001) and high ACh hydrolysis (Ferreira et al., 2012). This could also explain the cognitive impairment prevention by the PHY, since the pharmacological use in AD treatment of this natural alkaloid is solely due to AChE inhibition (Coelho and Birks, 2001). We also attribute this NAC beneficial effect to it capacity to prevent the mitochondrial dysfunction induced by the STZ (Costa et al., 2016), normalizing the synaptic plasticity in mice. The directly effects of NAC supplementation improving mitochondrial energy production efficiency was already described by Cocco et al. (2005). In this way, we conclude that the NAC treatment prevented the cognitive impairment induced by STZ in mice, restoring the cholinergic and synaptic plasticity in cerebral cortex and hippocampus.

Conflict of interest

The authors disclose no actual or potential conflicts of interest, including financial, personal, or relationships with other people or organizations. All authors have contributed to the work and agreed with the presented findings.

Acknowledgements

This work was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (http://www.capes.gov.br/), Conselho Nacional de Desenvolvimento Científico e Tecnológico (http://cnpq.br/) and Fundação para a Ciência e Tecnologia (http://www.fct.pt/).

References

Acheson, A., Conover, J.C., Fandl, J.P., DeChiara, T.M., Russell, M., Thadani, A., Squinto, S.P., Yancopoulos, G.D., Lindsay, R.M. 1994. A BDNF autocrine loop in adult sensory neurons prevents cell death. Nature 374, 450-453.

Agrawal, R., Mishra, B., Tyagi, E., Nath, C., Shukla, R. 2010. Effect of curcumin on brain insulin receptors and memory functions in STZ (ICV) induced dementia model of rat. Pharmacol. Res. 61, 247-252.

Agrawal, R., Tyagi, E., Shukla, R., Nath, C. 2009. A study of brain insulin receptors, AChE activity and oxidative stress in rat model of ICV STZ induced dementia. Neuropharmacology. 56, 779-787.

Azevedo, E., Mendes, A.C., Berk, M., Brietzke, E. 1999. To conduct the first systematic literature review of clinical trials of N-acetylcysteine (NAC) for the treatment of

substance abuse disorders and addictive behaviors. Rev. Bras. Psiquiatr. 36, 168-175.

Bartolini, L., Casamenti, F., Pepeu, G. 1996. Aniracetam restores object recognition impaired by age, scopolamine, and nucleus basalis lesions. Pharmacol. Biochem. Behav. 53, 277-283

Bekinschtein, P., Cammarota, M., Katche, C., Slipczuk, L., Rossato, J.I., Goldin, A., Izquierdo, I., Medina, J.H. 2008. BDNF is essential to promote persistence of long-term memory storage. Proc. Natil. Acad. Sci. U.S.A. 105, 2711-2716.

Bliss, T.V., Lomo, T. 1973. Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetized rabbit following stimulation of the performant path. J. Physiol. 232, 331-356.

Brentjens, R., Saltz, L. 2001. Islet cell tumors of the pancreas: the medical oncologist's perspective. Surg. Clin. North. Am. 81, 527-542

Calhoun, M.E., Jucker, M., Martin, L.J., Thinakaran, G., Price, D.L., Mouton, P.R. 1996. "Comparative evaluation of synaptophysin-based methods for quantification of synapses". J. Neurocytol. 25, 821-828.

Cocco, T., Sgobbo, P., Clemente, M., Lopriore, B., Grattagliano, I., Di Paola, M., Villani, G. 2005. Tissue-specific changes of mitochondrial functions in aged rats: Effect of a long-term dietary treatment with N-acetylcysteine. Free Radic. Biol. Med. 38, 796-805.

Coelho, F., Birks, J. 2001. Physostigmine for Alzheimer's disease. Cochrane Database Syst. Rev. CD001499.

Costa, M., Bernardi, J., Fiuza, T., Costa, L., Brandão, R., Pereira, M.E. 2016. N-acetylcysteine protects memory decline induced by streptozotocin in mice. Chem. Biol. Interact. 253, 10-17.

D'Hooge, R., Deyn, P. 2001. Applications of the Morris water maze in the study of learning and memory. Brain Res. Rev. 36, 60-90.

de la Monte, S.M., Wands, J.R. 2008. Alzheimer's Disease Is Type 3 Diabetes-Evidence Reviewed. J. Diabetes Sci. Technol. 2, 1101-1113.

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

Ferreira, G.K., Carvalho-Silva, M., Gonçalves, C.L., Vieira, J.S., Scaini, G., Ghedim, F.V., Deroza, P.F., Zugno, A.I., Pereira, T.C., Oliveira, G.M., Kist, L.W., Bogo,

M.R., Schuck, P.F., Ferreira, G.C., Streck, E.L. 2012. L-Tyrosine administration increases acetylcholinesterase activity in rats. Neurochem. Int. 61, 1370-1374

Figueiró, M., Ilha, J., Pochmann, D., Porciúncula, L.O., Xavier, L.L., Achaval. M., Nunes, D.S., Elisabetsky, E. 2010. Acetylcholinesterase inhibition in cognition-relevant brain areas of mice treated with a nootropic Amazonian herbal (Marapuama). Phytomedicine. 17, 956-962.

Francis, P.T., Palmer, A.M., Snape, M., Wilcock, G.K. 1999. The cholinergic hypothesis of Alzheimer's disease: a review of progress. J. Neurol. Neurosurg. Psychiatry. 66,137-147.

Gomes, F.C.A., Paulin, D., Moura, V. 1999. Glial fibrillary acidic protein (GFAP): modulation by growth factors and its implication in astrocyte differentiation. Effects of growth factors on GFAP expression. Braz. J. Med. Biol. Res. 32, 619-631.

Haley, T.J., McCormick, W.G. 1957. Pharmacological effects produced by intracerebral injection of drugs in the conscious mouse. Br. J. Pharmacol. Chemother. 12, 12-15.

Holden, M., Kelly, C. 2002. Use of cholinesterase inhibitors in dementia, Adv. Psychiatr. Treat. 8, 89-96.

Huang. E.J., Reichardt. L.F. 2001. Neurotrophins: Roles in Neuronal Development and Function. Ann. Rev. Neurosci. 24, 677-736.

Ito, D., Tanaka, K., Suzuki, S., Dembo, T., Fukuuchi, Y. 2001. Enhanced expression of Iba1, ionized calcium-binding adapter molecule 1, after transient focal cerebral ischemia in rat brain. Stroke. 32, 1208-1215.

Janssen, B., Vugts, D.J., Funke, U., Molenaar, G.T., Kruijer, P.S., van Berckel, B.N., Lammertsma, A.A., Windhorst. A.D. 2016. Imaging of neuroinflammation in Alzheimer's disease, multiple sclerosis and stroke: Recent developments in positron emission tomography. Biochim. Biophys. Acta. 1862, 425-441.

Johnston-Wilson, N.L., Sims, C.D., Hofmann, J.P., Anderson, L., Shore, A.D., Torrey, E.F., Yolken, R.H. 2000. "Disease-specific alterations in frontal cortex brain proteins in schizophrenia, bipolar disorder, and major depressive disorder. The Stanley Neuropathology Consortium". Molec. Psyc. 5, 142-149

Klinkenberg, I., Sambeth, A., Blokland, A. 2011. Acetylcholine and attention. Behav. Brain Res. 221, 430-442.

Konzack, A., Jakupovic, M., Kubaichuk, K., Görlach, A., Dombrowski, F., Miinalainen, I., Sormunen, R., Kietzmann, T. 2015. Mitochondrial Dysfunction Due to Lack of

Manganese Superoxide Dismutase Promotes Hepatocarcinogenesis. Antioxid. Redox Signal. 10, 1059-1075.

Kristensen, M. 1990. Neurotransmitters in Alzheimer's disease. Ugeskr. Laeger. 152, 2165-2168.

Ladrech, S., Lenoir, M., Ruel, J., Puel, J. 2003. Microtubule-associated protein 2 (MAP2) expression during synaptic plasticity in the guinea pig cochlea. Hear. Res. 186, 85-90.

Lane, R.M., Kivipelto, M., Greig, N.H., 2004. Acetylcholinesterase and its inhibition in Alzheimer disease. Clin. Neuropharmacol. 27, 141-149.

Lannert, H., Hoyer, S. 1998. Intracerebroventricular administration of streptozotocin causes long-term diminutions in learning and memory abilities and in cerebral energy metabolism in adult rats. Behav Neurosci. 112, 1199-1208.

Laursen, S.E., Belknap, J.K. 1986. Intracerebroventricular injections in mice: some methodological refinements. J. Pharmacol. Methods. 16, 355-357.

Lester-Coll, N., Rivera, E.J., Soscia, S.J., Doiron, K., Wands, J.R., de la Monte, S.M. 2006. Intracerebral streptozotocin model of type 3 diabetes: Relevance to sporadic Alzheimer's disease. J. Alzheimers Dis. 9,13-33.

Lu, Y., Christian, K., Lu, B. 2008. BDNF: a key regulator for protein synthesis dependent LTP and long term memory. Neurobiol. Learn. Mem. 89, 312-323.

Mach, M., Grubbsa, R.D., Pricea, W.A., Patona, S.J., Lucot. J.B. 2004. Behavioral changes after acetylcholinesterase inhibition with physostigmine in mice. Pharmacol. Biochem. Behav. 79, 533-540

Morris, R. 1984. Developments of a water-maze procedure for studying spatial-learning in the rat. J. Neurosci. Methods. 11, 47-60.

Morris, R.G.M., Garrud, P., Rawlins, J.N.P., O'keefe, J. 1982. Place navigation impaired in rats with hippocampal lesions. Nature. 297, 681-683.

Osborn, L.M., Kamphuis, W., Wadman, W.J., Hol, E.M. 2016. Astrogliosis: An integral player in the pathogenesis of Alzheimer's disease. Prog. Neurobiol. pii: S0301-0082(15)30021-6.

Pekny, M., Pekna, A. 2004. Astrocyte intermediate filaments in CNS pathologies and regeneration. J. Pathol. 204, 428-437.

Pinton, S., Brüning, C.A., Oliveira, C.S., Prigol, M., Nogueira, C.W. 2013. Therapeutic effect of organoselenium dietary supplementation in a sporadic dementia of Alzheimer's type model in rats. J. Nutr. Biochem. 24, 311-317.

Pitsikas, N., Markou, A. 2014. The metabotropic glutamate 2/3 receptor agonist LY379268 counteracted ketamine-and apomorphine-induced performance deficits in the object recognition task, but not object location task, in rats. Neuropharmacology. 85, 27-35.

Plaschke, K., Hoyer, S. 1993. Action of the diabetogenic drug streptozotocin on glycolytic and glycogenolytic metabolism in adult rat brain cortex and hippocampus, Int. J. Dev. Neurosci. 11, 477-483.

Prakash, A., Kaira, J.K., Kumar, A. 2015. Neuroprotective effect of N-acetyl cysteine against streptozotocin-induced memory dysfunction and oxidative damage in rats. J. Basic. Clin. Physiol. Pharmacol. 26, 13-23.

Resende, R., Pereira, C., Agostinh,o P., Vieira, A.P., Malva, J.O., Oliveira, C.R. 2007. Susceptibility of hippocampal neurons to A peptide toxicity is associated with perturbation of Ca 2+ homeostasis. Brain Res. 11, 11-21

Saez-Valero, J., Fodero, L.R., White, A.R., Barrow, C.J., Small, D.H. 2003. Acetylcholinesterase is increased in mouse neuronal and astrocyte cultures after treatment with β-amyloid peptides. Brain Research. 965, 283-286.

Saxena, G., Patro, I.K., Nath, C. 2011. ICV STZ induced impairment in memory and neuronal mitochondrial function: A protective role of nicotinic receptor. Behav. Brain Res. 224, 50-57.

Shelton, M.A., Newman, J.T., Gu, H., Sampson, A.R., Fish, K.N., MacDonald, M.L, Moyer, C.E., DiBitetto, J.V., Dorph-Petersen, K., Penzes, P., Lewis, D.A., Sweet, R.A. 2015. Loss of Microtubule-Associated Protein 2 Immunoreactivity Linked to Dendritic Spine Loss in Schizophrenia. Biol. Psychiatry. 78, 374-385

Siddiqui, M.F., Levey, A.I. 1999. Cholinergic therapies in Alzheimer's disease. Drugs Future. 24, 417-444

Sofroniew, M.V., Vinters, H.V., Astrocytes: biology and pathology. 2010. Acta Neuropathol. 119, 7-35.

Suzuki, K., Ota, H., Sasagawa, S., Sakatani, T., Fujikura, T. 1983. Assay method for myeloperoxidase in human polymorphonuclear leukocytes. Anal. Biochem. 132, 345-352.

Sze, C., Troncoso, J.C., Kawas, C., Mouton, P., Price, D.L, Martin, L.J. 1997. Loss of the presynaptic vesicle protein synaptophysin in hippocampus correlates with cognitive decline in Alzheimer disease. J. Neuropathol. Exp. Neurol. 56, 933-944.

Talesa, V.N. 2001. Acetylcholinesterase in Alzheimer's disease. Mech. Ageing Dev. 122, 1961-1969.

Tota, S., Kamat, P.K., Saxena, G., Hanif, K., Najmi, A.K., Nath, C. 2012. Central angiotensin converting enzyme facilitates memory impairment in intracerebroventricular streptozotocin treated rats, Behav. Brain Res. 226, 317-730.

Verkhratsky, A., Butt, A. 2013. Glial Physiology and Pathophysiology. John Wiley & Sons, Ltd, The Atrium, Southern Gate, Chichester, West Sussex, UK. Chapter 4.

Verkhratsky, A., Olabarria, M., Noristani, H., Yeh, C., Rodriguez, JJ. 2010. Astrocytes in Alzheimer's Disease. NeuroTherapeutics. 7, 399-412.

Walsh, R.N., Cummins, R.A. 1976. The open-field test: a critical review. Psychol. Bull. 83, 482-504.

WHO Model List of Essential Medicines. 2014. World Health Organization.

Yamada, K., Nabeshima, T. 2003. Brain-derived neurotrophic factor/TrkB signaling in memory processes. J. Pharmacol. Sci. 91, 267-270.

Zunino, G., Messina, A., Sgadò, P., Baj, G., Casarosa, S., Bozzi. Y. 2016. Brain-derived neurotrophic factor signaling is altered in the forebrain of Engrailed-2 knockout mice. Neuroscience. 324, 252–261.

4 DISCUSSÃO

A memória é a faculdade de conservar e readquirir ideias ou imagens de consciência passada, e tudo que seja associada as mesmas (MICHAELIS, 2009). Considerando-se assim, algo muito importante para o indivíduo. Uma vez que, a vida se tornaria mais difícil caso não fosse possível recordar certas coisas, como nomes, cheiros, locais ou ações entre outras tantas. Contudo, esta é a realidade de pessoas que possuem DA, a qual tem como principal característica a perda progressiva e irreversível da memória. Para o ano de 2012 estimavam-se cerca de 35,5 milhões de pessoas com este tipo de demência no mundo. Acredita-se que para o ano de 2030 sejam cerca de 65,7 milhões, e de 115,4 milhões para o ano de 2050 (WHO, 2012). Já no Brasil, de acordo com dados do Instituto Brasileiro de Geografia e Estatística (IBGE), para o ano de 2012 acreditava-se que cerda de 1,2 milhões de pessoas sofriam com a DA, e de que este número aumentaria em cerca de 100 mil novos casos por ano (IBGE, 2012). Chegando desta forma, a aproximadamente 1,6 milhões de pessoas no presente ano.

Além da perda progressiva da memória, a DA também se caracteriza por uma série de alterações genéticas, neurológicas e neurofisiológicas (ALMEIDA, 1997). Como mencionado anteriormente, uma das hipóteses para o surgimento do déficit cognitivo e consequente perda da memória na DA é a hipótese colinérgica. Foi com base nesta, que se desenvolveram estratégias para a terapia da doença focadas em melhorar a hipofunção colinérgica. Dentre elas, destaca-se a utilização de fármacos que possam vir a influenciar na atividade da AChE (os iAChE). Nos anos 80 esta era a estratégia a qual tinha mostrado uma maior eficácia no tratamento da doença (PERRY e col., 1978), e continuou sendo assim nas décadas seguintes (GIACOBINI, 1996; OSVALDO, 1998; HOLDEN & KELLY, 2002; INOUYE & DE OLIVEIRA, 2004). Contudo, devido ao fato de hoje em dia ainda não existir cura para a DA, o objetivo maior é, o desenvolvimento de novas terapias para o tratamento sintomático da doença (PINTON, 2012). Com base nestes dados, o objetivo do presente trabalho consistiu em avaliar o efeito neuroprotetor da NAC no déficit cognitivo induzido pela STZ em camundongos.

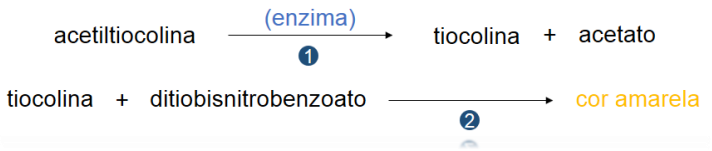
Os resultados apresentados aqui demonstraram que a NAC interferiu na atividade da AChE cerebral de camundongos *in vitro*, inibindo a atividade desta enzima de uma forma não-competitiva[49]. Já *in vivo*, a administração via oral de NAC por um período curto (de 9 dias), assim como, por um período maior (de 30 dias) se demonstrou

[49] Tipo de inibição enzimática onde o inibidor reduz a atividade enzimática ao ligar-se tanto à enzima quanto ao complexo enzima-substrato (STRELOW e col., 2012).

eficaz na prevenção das deteriorações das memórias de curta e de longa duração, como também, da aprendizagem, induzidas pela injeção i.c.v. de STZ em camundongos[50]. Principalmente prevenindo o aumento na atividade das ChEs e a diminuição da ACh no córtex cerebral, hipocampo e estriado.

Em um primeiro momento, procurou-se avaliar o efeito da NAC sobre a atividade da AChE cerebral de camundongos (protocolo 1), para posteriormente proceder-se à sua utilização em modelos experimentais de comprometimento cognitivo (protocolos 2 e 3). Não são poucas as metodologias existentes hoje em dia para a mensuração da atividade da AChE em homogeneizados. Porém, a mais utilizada continua sendo a técnica colorimétrica de George Ellman, Diane Courtney, Valentino Andres e Robert Featherstone publicada em 1961 (ELLMAN e col., 1961). Esta se baseia na determinação da atividade enzimática pela mensuração do produto de cor amarela formado da interação do cromogênio ditiobisnitrobenzoato (DTNB) e a tiocolina (Figura 9). Contudo, esta metodologia é limitada quando se trata de testar antídotos contra iAChE, para medir a atividade enzimática em amostras de indivíduos tratados com iAChE (SINKO e col., 2007) e quando há presença de compostos que possam interagir com os substratos, já poderia obter-se resultados falsos positivos.

Figura 9. Método colorimétrico para determinação da AChE.



Em uma primeira reação (1) a acetiltiocolina (ATCh - análogo da acetilcolina) é hidrolisada pela enzima produzindo tiocolina e acetato, logo após, em uma segunda reação (2) a tiocolina reage com o ditiobisnitrobenzoato (DTNB) formando o tionitrobenzoato (TNB^-) o qual possui uma coloração amarela.

Assim, para proceder-se ao estudo do efeito da NAC na atividade catalítica da AChE utilizando-se da metodologia de Ellman e seus colaboradores, algumas adequações a esta foram necessárias. Assim, inicialmente foram estabelecidas a concentração de DTNB e o pH ideais para a reação enzimática. A linearidade da reação para cada concentração de ATCh também foi determinada. Um tempo de estabilização foi adicionado à metodologia, uma vez que falsos resultados poderiam ser consequência da escassez de substrato para a reação, devido ao fato de que a NAC possui em sua

[50] Ver protocolos de administração *in vivo* 2 e 3 em Apêndice 1.

estrutura um grupamento tiol (-SH), o qual reage de forma direta com o DTNB. Desta forma, optou-se pelo tempo o qual era necessário para que a maior concentração de NAC reagisse com o DTNB, uma vez que o tempo de reação entre estes é diretamente proporcional à concentração de NAC. Contudo, sendo de suma importância também de que, a concentração de DTNB utilizada fosse o suficiente para interagir com a tiocolina após em parte ter reagido com a NAC. Assim, após serem estabelecidas as condições ideais para a reação enzimática, procedeu-se ao estudo cinético da AChE.

O estudo da cinética de uma enzima permite elucidar os pormenores do seu mecanismo catalítico, em especial a velocidade de reação e como esta pode ser inibida ou potenciada por outras moléculas. A NAC inibiu a atividade da AChE de uma maneira não-competitiva, uma vez que a atividade enzimática diminuiu de forma dependente da concentração de NAC utilizada e não foram observadas diferenças nas K_m[51]. Este foi um resultado muito promissor, uma vez que, os iAChE não-competitivos são utilizados no tratamento de diversas demências, como por exemplo a DA (HOLDEN e KELLY, 2002). Um potencial mecanismo para explicar o efeito inibitório da NAC sobre a AChE foi proposto (ver esquema 2 do protocolo 1; pág. 48). Contudo, é claro que outras hipóteses não foram descartadas. Uma das características dos inibidores não-competitivos é que estes ligam-se a sítios alostéricos e não ao sítio ativo da enzima (STRELOW e col., 2012). Os resultados obtidos, também demonstraram uma possível ligação alostérica à AChE por parte da NAC, o qual poderia levar a uma alteração conformacional e consequente perda da atividade enzimática. Esta inibição da enzima não foi dependente do tempo de incubação com NAC, demonstrando assim a irreversibilidade da inibição.

Os resultados apresentados nos protocolos 2 e 3, demonstram que a injeção i.c.v. de STZ prejudicou a capacidade de aprendizagem e a memória dos animais, como pôde ser observado nos testes do labirinto aquático (MWM), da esquiva passiva (SDPA) e do reconhecimento do novo objeto (NOR). Este prejuízo na cognição foi visível a partir do sétimo dia após a administração da STZ (protocolo 2) e estendeu-se por um longo período (protocolo 3). A injeção i.c.v. de STZ é um modelo de demência esporádica de DA, e desta forma poderia levar ao comprometimento da capacidade motora, também caraterística desta doença (MARK e col., 2015). Contudo, nenhum animal apresentou sinais de comprometimento motor, como pôde ser observado no teste de campo aberto (OF) (protocolos 2 e 3). Assim, descartamos a interferência deste fator nos resultados

[51] Constante de Michaelis-Menten: concentração de substrato para a qual a velocidade da reação enzimática é metade da velocidade máxima.

obtidos nos testes comportamentais de cognição. O tratamento com NAC preveniu este prejuízo na aprendizagem e memória induzido pela STZ em todos os testes cognitivos aqui realizados.

Sintomas neuropsiquiátricos como psicose, e alterações do humor como depressão, apatia e agitação podem estar presentes durante o curso da DA. Estas perturbações geralmente refletem alterações estruturais e neuroquímicas de áreas cerebrais como o córtex e o hipocampo (WOLTJER e MILATOVIC, 2006). Os neurónios corticais e hipocampais são inervados pelos aferentes[52] colinérgicos do núcleo basal, onde encontram-se aproximadamente 80% dos neurônios colinérgicos do SNC e onde também é observada uma grande atrofia em pacientes com DA (CUMMINGS e BACK, 1998). Desta forma, acredita-se que falhas no sistema colinérgico possam contribuir com os sintomas observados na DA. Um aumento nas atividades da AChE e BChE no córtex cerebral e no hipocampo (protocolos 2 e 3) e consequente diminuição dos níveis de ACh (protocolo 2) nestas áreas foram observadas nos animais que receberam a STZ. Isto levou a uma redução na transmissão colinérgica, contribuindo assim para o prejuízo na capacidade de aprendizagem e na memória desses animais (HASSELMO, 2006). A redução nos níveis de ACh também pode ter sido aqui, consequência da degeneração dos neurônios colinérgicos.

O uso de iAChE inicialmente veio à atenção dos investigadores após a descoberta da depleção da ACh hipocampal em modelos de DA (SMITH e SWASH, 1978) e dos efeitos benéficos da fisostigmina (PHY) na cognição, em sujeitos normais (DAVIS e col., 1978) e em pacientes com DA (MURAMOTO e col., 1979). Os animais que receberam a injeção i.c.v. de STZ, e também receberam o tratamento com NAC, não apresentaram aumento nas atividades da AChE e BChE no córtex cerebral e hipocampo (protocolos 2 e 3). Apesar da NAC ter inibido a atividade da AChE *in vitro* (protocolo 1) a dose utilizada nos experimentos *in vivo* (protocolos 2 e 3) não afetou *per se* a atividade da enzima, uma vez que uma dose subefetiva foi escolhida. Por outro lado, o tratamento com NAC recuperou os níveis de ACh no córtex cerebral, hipocampo e estriado nos animais que receberam a STZ. A melhora na transmissão colinérgica como consequência disto, refletiu no melhor desempenho apesentado por esses animais nos testes comportamentais quando comparados aos animais que somente receberam a injeção i.c.v. de STZ.

Na DA, a deterioração cognitiva está associada à perda neuronal, principalmente no córtex cerebral e hipocampo (RESENDE e col., 2007). A injeção i.c.v. de STZ pode

[52] Nervos que levam aos centros nervosos, os impulsos originados nos receptores periféricos.

causar danos neuronais, provocando prejuízo na utilização da glicose no cérebro (SAXENA e col., 2010). Uma instabilidade do metabolismo energético cerebral foi observada nos animais que receberam a injeção i.c.v. de STZ. Uma vez que, apresentaram uma menor captação de glicose no hipocampo (protocolo 2), indicando assim, uma menor utilização do carboidrato. Além disso, níveis cerebrais de adenosina trifosfato (ATP) inferiores e de adenosina (ADO) elevados (protocolo 2) também foram observados nestes. Somando a isto, menores níveis da enzima mitocondrial superóxido dismutase dependente de manganês (MnSOD) foram encontrados no córtex cerebral destes animais (protocolo 3). Danos mitocondriais representam eventos precoces na patogênese da DA (KEDAR e col., 2002) os quais podem acarretar distúrbios no metabolismo energético. Os animais que receberam o tratamento com a NAC apresentaram níveis semelhantes aos controles em relação a captação de glicose e tiveram em parte a prevenção do decréscimo nos níveis de ATP. Esta melhora na taxa de utilização da glicose pode estar relacionada com uma maior síntese e liberação de ACh (HENNEBERG e HOYER, 1995), como observada no protocolo 2. Estes resultados corroboram estudos de Cocco e col. (2005), que demonstraram uma melhora na produção de energia mitocondrial devido à suplementação com NAC.

O dano neuronal provocado pela STZ também causou uma perda da plasticidade sináptica (protocolo 3) como consequência da diminuição dos níveis dos receptores tirosina quinase B fosforilado (pTrkB)[53] e da sinaptofisina (SYN)[54] corticais e hipocampais, já que o pTrkB está implicado na diferenciação e sobrevivência neuronal, e juntamente com a SYN, participa no processo de sinaptogênese e nos mecanismos da plasticidade sináptica relacionados à memória no SNC (CUNHA e col., 2010). Por outro lado, o mesmo não foi observado nos animais que receberam o tratamento com NAC. A manutenção dos níveis destas duas proteínas, refletiu nos resultados obtidos pelos animais nos testes comportamentais de memória, demonstrando assim a capacidade neuroprotetora do composto. Acredita-se que, a perda da plasticidade sináptica possa ter sido consequência da instabilidade do metabolismo energético. Uma vez que a liberação de ATP na fenda sináptica está relacionada com a formação da memória durante a potenciação de longa duração (LTP)[55] (RAYMOND e col., 1993). Desta forma, o efeito neuroprotetor da NAC pode aqui ser atribuído em parte, a sua

[53] Do inglês "*Tropomyosin receptor kinase B"*.: É o receptor catalítico do fator neurotrófico derivado cerebral (do inglês *Brain-derived neurotrophic fator*: BDNF). A forma fosforilada do receptor (pTrkB) indica de forma indireta a quantidade da neurotrofina presente.
[54] Do inglês "*Synaptophysin"*.: Glicoproteína sináptica presente em células neuroendócrinas
[55] Do inglês "*Long-term potentiation"*.: É um reforço persistente das sinapses com base em recentes padrões de atividade. Estes padrões produzem um aumento de longa duração nas transmissões de sinais entre neurônios (COOKE e BLISS, 2006).

possível participação na capacidade bioenergética mitocondrial (MARTÍNES e col., 1999), já que grupamentos -SH são essenciais para a cadeia transportadora de e^- (MARTINES, 2000).

Além da diminuição dos níveis de pTrkB e SYN, a STZ também provocou uma diminuição nos níveis da proteína associada aos microtúbulos do tipo 2 (MAP2)[56] no córtex cerebral e hipocampo, ressaltando desta forma o dano neuronal e a perda da plasticidade sináptica (SÁNCHES e col., 2000) provocados pela STZ. O tratamento com NAC protegeu da diminuição dos níveis de MAP2, sugerindo a participação da NAC na diferenciação neuronal e a neuritogênese. A estimulação da expressão da MAP2 pela NAC já tinha sido demonstrada por Quian e Yang (2009), em um estudo *in vitro*. Outras células da neuroglia como os astrócitos (VERKHRATSKY e col., 2010) e microgliócitos (JANSSEN e col., 2016) também são afetados na DA. A injeção i.c.v. de STZ provocou uma diminuição nos níveis da proteína ácida fibrilar glial (GFAP)[57] o qual contribuiria para a progressão do déficit cognitivo. O tratamento com NAC atenuou a diminuição dos níveis de GFAP, possivelmente devido a seu efeito na proteção do decréscimo no metabolismo energético, uma vez que isto levaria à neurodegeneração[58]. É de ser notar que os níveis de GFAP diminuíram no cortex cerebral e hipocampo dos animais tratados com STZ, uma vez que, o aumento nos níveis desta proteína está relacionado à neuroinflamação, um fenômeno característico na DA (RODRÍGUEZ-ARELLANO e col., 2016). Além do mais, os níveis da molécula adaptadora ligante de cálcio ionizado-1 (IBA1)[59] também não foram afetados pela STZ, indicando assim a ausência do processo inflamatório.

Respostas inflamatórias mediadas principalmente pela micróglia são características da patologia da DA (HEPPNER e col., 2015). Contudo, além de decréscimo nos níveis de GFAP e de não terem sido observadas alterações nos níveis da IBA1 no protocolo 3, também não foram observadas alterações na atividade da mieloperoxidase (MPO)[60] no córtex cerebral, hipocampo e estriado no protocolo 2 (Apêndice 2) nos animais que receberam STZ. Outra característica desta patologia é o surgimento do EO, o qual também está relacionado ao processo de neuroinflamação na DA (POHANKA 2013). Somando-se a isso, relatos do envolvimento do EO no modelo

[56] Do inglês "*Microtubule-associated protein 2"*.: Família de proteínas abundante no citoesqueleto dos neurónios, com participação no processo da montagem dos microtúbulos durante a neuritogênese.
[57] Do inglês "*Glial Fibrillary Acidic Protein"*.: Proteína fibrilar presente nos filamentos intermediários do citoesqueleto dos astrócitos, com participação na comunicação astrócitos-neurônio e neurogênese.
[58] Hiperônimo para a perda progressiva da estrutura ou funcionamento dos neurônios.
[59] Do inglês "*Ionized calcium-binding adapter molecule 1"*.: Proteína citoplasmática expressa especificamente na micróglia, com participação na reparação do tecido nervoso danificado (ITO e col., 1998).
[60] Enzima constituinte dos neutrófilos, participa na geração de espécies reativas de oxigênio e nitrogênio, e na condução do processo inflamatório (WANG e col., 2014).

de déficit cognitivo induzido pela injeção i.c.v. de STZ também são encontrados na literatura (SHARMA e GUPTA, 2001; JAVED e col., 2011). No entanto, a capacidade antioxidante total no tecido cerebral não foi afetada nos animais que receberam a injeção i.c.v de STZ, como pôde ser visto nos níveis de tióis totais (TSH) e tióis não-proteicos (NPSH) e na capacidade de neutralizar o radical ABTS[61] no protocolo 2 (Apêndices 3 e 4). É um fato de que a infrarregulação[62] do metabolismo energético na DA, e como a observada também neste trabalho, tem sido considera como uma consequência do dano mitocondrial devido ao EO (JIVA e col., 2012). Desta forma, a inexistência de uma resposta ao aumento do EO, assim como para o processo inflamatório, poderiam sugerir o contrário. Esta, é a questão central no domínio da doença de Alzheimer (DA), a de separar a causa da consequência entre muitas características patológicas observadas (JIVA e col., 2012). A inexistência destes também possa ser devido à dose de STZ utilizada, uma vez que lesões neuronais consequentes da inflamação e excessiva produção de EO são associadas a doses maiores a 1 mg/kg (GRIEB, 2016). Contudo, não é descartada a possibilidade da participação destes dois processos no déficit cognitivo induzido aqui pelo STZ, já que mais testes seriam necessários para excluir esta hipótese, assim como, de uma possível resposta a estes, por parte da NAC, uma vez que esta apresenta propriedades antioxidantes e anti-inflamatórias (ASEVEDO, 2014).

[61] Sal de amônio do ácido 2,2'-azinobis(3-etilbenzenotiazolina- 6-sulfônico)
[62] Do inglês "*Downregulation*".: Processo no qual há o decréscimo de algum componente celular em resposta à uma variável externa.

5 CONCLUSÕES

Com os resultados apresentados nesta tese, pode-se concluir que:

✓ A NAC inibiu de forma não-competitiva a atividade da AChE cerebral de camundongos *in vitro*;

✓ A NAC protegeu do prejuízo cognitivo, aumentando a atividade da AChE e BChE cerebrais e, do decréscimo dos níveis de ACh induzidos pela STZ;

✓ A NAC protegeu da neurodegeneração e perda de plasticidade sináptica induzida pela STZ;

✓ Desta forma, o tratamento com NAC foi eficaz na prevenção do déficit cognitivo e das alterações bioquímicas induzidas pela STZ em camundongos.

REFERÊNCIAS

AARSLAND D., MOSIMANN U.E., MCKEITH I.G. **Role of cholinesterase inhibitors in Parkinson's disease and dementia with Lewy bodies**. J Geriatr Psychiatr Neurol 17 (2004) 164-171.

ADLER M., MANLEY H.A., PURCELL A.L., DESHPANDE S.S., HAMILTON T.A., KAN R.K., OYLER G., LOCKRIDGE O., DUYSEN E.G., SHERIDAN R.E. **Reduced acetylcholine receptor density, morphological remodeling, and butyrylcholinesterase activity can sustain muscle function in acetylcholinesterase knockout mice.** Musc Nerve 30 (2004) 317-327.

ALDRIDGE W.N., REINER E. **Enzyme Inhibitors as Substrates**. North-Holland/American (1972) Elsevier, Amsterdam.

ALMEIDA O.P. **Biologia molecular da doença de Alzheimer: Uma luz no fim do túnel?** Rev Assoc Med Bras 43 (1997) 77-81.

ALMEIDA OP. **Tratamento da doença de Alzheimer, Avaliação crítica sobre o uso de anticolinesterásicos** Arq Neuropsiq 56 (1998) 688-696.

ANDERSON R.B., KEY B. **Role of acetylcholinesterase in the development of axon tracts within the embryonic vertebrate brain.** Int J Devel Neurosci 17 (1999) 787-793.

ASEVEDO E., MENDES A.C., BERK M., BRIETZKE E. **Systematic review of N-acetylcysteine in the treatment of addictions.** Rev Bras Psiq 36 (2014) 168-175.

ASEVEDO E., MENDES A.C., BERK M., BRIETZKE E. **Systematic review of N-acetylcysteine in the treatment of addictions**. Rev Bras Psiquiatr 36 (2014) 168-175.

AULD D.S., KORNECOOK T.J., BASTIANETTO S., QUIRION R. **Alzheimer's disease and the basal forebrain cholinergic system.** Prog Neurobiol 68 (2002) 209-245.

BARTUS R. T. **Evidence for a direct cholinergic involvement in the scopolamine-induced amnesia in monkeys: Effects of con- current administration of physostigmine and methylphenidate with scopolamine.** Pharmacol Biochem Behav 9 (1978) 833-836.

BECKER R.E., COLLIVER J.A., MARKWELL S.J., MORIEARTY P.L., UNNI L.K., VICARI S. **Effects of metrifonate on cognitive decline in Alzheimer disease: a blouble-blind, placebo-controlled, 6-month study.** Alzh Dis Assoc Disord 12 (1998) 54-57.

BERK M., MALHI G.S., GRAY L.J., DEAN O.M. **The promise of N-acetylcysteine in neuropsychiatry**. Trends in Pharmacol Sci 34 (2013) 167-77.

BRIMIJOIN S. **Enzymology and biology of cholinesterases.** In "Proceedings of the U.S. EPA Workshop on Cholinesterase Methodologies" pp. 30. U.S. Environmental Protection Agency (1992) Washington, DC.

CAI Y., CRONIN C.N., ENGEL A.G., OHNO K., HERSH L.B., RODGERS D.W. **Choline acetyltransferase structure reveals distribution of mutations that cause motor disorders**. EMBO J 23 (2004) 2047-2058.

CHEN X., FANG L., LIU J., ZHAN C. **Reaction Pathway and Free Energy Profile for Butyrylcholinesterase-Catalyzed Hydrolysis of Acetylcholine.** J Phys Chem B 115 (2011) 1315-1322.

COCCO T., SGOBBO P., CLEMENTE M., LOPRIORE B., GRATTAGLIANO I., DI PAOLA M., VILLANI G. **Tissue-specific changes of mitochondrial functions in aged rats: Effect of a long-term dietary treatment with N-acetylcysteine.** Free Radic Biol Med. 38 (2005) 796-805.

COOKE S.F., BLISS T.V. **"Plasticity in the human central nervous system".** Brain 129 (2006) 1659-1673.

COREN S., LAWRENCE M.W., JAMES T. **Sensation and Perception**. Harcourt (1999) 9.

COUSIN X., BON S., MASSOULIE J., BON C. **Identification of a Novel Type of Alternatively Spliced Exon from the Acetylcholinesterase Gene of Bungarus fasciatus. Molecular forms of acetylcholinesterase in the snake liver and muscle.** J Biol Chem 273 (1998) 9812-9820.

CUMMINGS J.L., BACK C. **The cholinergic hypothesis of neuropsychiatric symptoms in Alzheimer's disease**. Am J Geriatr Psychiatr 6 (1998) 64-78.

CUNHA C., BRAMBILLA R., THOMAS K.L. **A simple role for BDNF in learning and memory?** Front Mol Neurosci. 3 (2010) 1-14.

DAJAS-BAILADOR E A., HEIMALA K., WONNACOTT S. **The allosteric potentiation of nicotinic acetylcholine receptors by galantamine is transduced into cellular responses in neurons: Ca 2+ signals and neurotransmitter release.** Mol Pharmacol 64 (2003) 1217-1226.

DARVESH S., WALSH R., KUMAR R. **Kinetic properties of human acetylcholinesterase and butyrylcholinesterase in the presence of drugs for Alzheimer's disease**. Alzheimer Dis Assoc Disord 17 (2003) 117-126.

DAVIGLUS M.L., BELL C.C., BERRETTINI W., BOWEN P.E., CONNOLLY E.S., COX N.J., DUNBAR-JACOB J.M., GRANIERI E.C., HUNT G., MCGARRY K., PATEL D., POTOSKY A.L., SANDERS-BUSH E., SILBERBERG D., TREVISAN M. **National Institutes of Health State-of-the-Science Conference Statement: Preventing Alzheimer's Disease and Cognitive Decline**. NIH Consens State Sci Statements. 4 (2010) 1-30

DAVIS K.L., MOHS R.C., TINKLENBERG J.R., PFEFFERBAUM A., HOLLISTER L.E., KOPELL B.S. **Physostigmine: Improvement of long-term memory processes in normal humans.** Science 201 (1978) 272-274.

DEKHUIJZEN P. **Antioxidant properties of N-acetylcysteine: their relevance in relation to chronic obstructive pulmonary disease.** Eur Respir J 23 (2004) 629-636.

DOBRANSKY T. RYLETT R.J. **Functional Regulation of Choline Acetyltransferase by Phosphorylation**. Neurochem Res 28 (2003) 537-542.

DODD S., MAES M., ANDERSON G., DEAN O.M., MOYLAN S., BERK M. **Putative neuroprotective agents in neuropsychiatric disorders.** Prog Neuropsychopharmacol Biol Psychiatry 42 (2013) 135-145.

DOOLEY M., LAMB H.M. **Donepezil: a review of its use in Alzheimer´s disease.** Drugs Aging 16 (2000) 199-226.

DUELLI R., SCHRÖCK H., KUSCHINSKY W., HOYER S. **Intracerebroventricular injection of streptozotocin induces discrete local changes in cerebral glucose utilization in rats.** Int J Dev Neurosci 12 (1994) 737–743.

ECOBICHON D. J. **Toxic effects of pesticides.** In Cass Doull's Toxicol 5th ed., (1996). McGraw-Hill, New York.

ELLMAN G.L., COURTNEY K.D., ANDRES JR.V., FEATHERSTONE R.M. **A new and rapid colorimetric determination of acetylcholinesterase activity**. Biochem Pharmacol 7 (1961) 88-95.

FERRI C.P., PRINCE M., BRAYNE C., BRODATY H., FRATIGLIONI L., GANGULI M., HALL K., HASEGAWA K., HENDRIE H., HUANG Y., JORM A., MATHERS C., MENEZES P.R., RIMMER E., SCAZUFCA M. **Global prevalence of dementia: a Delphi consensus study**. Lancet 366 (2005) 2112-2117.

FRÖLICH L., BLUM-DEGEN D., BERNSTEIN H.G., ENGELSBERGER S., HUMRICH J., LAUFER S., MUSCHNER D., THALHEIMER A., TURK A., HOYER S., ZOCHLING R., BOISSL K.W., JELLINGER K., RIEDERER P. **Brain insulin and insulin receptors in aging and sporadic Alzheimer's disease.** J Neur Trans105 (1998) 423-428.

FUCHS E., WEBER K. **Intermediate Filaments: Structure, Dynamics, Function and Disease.** Annu Rev Biochem 63 (1994) 345-382.

FURLONG C.E., LI W.E., RICHTER R.J., SHIH D.M., LUSIS A.J., ALLEVA E., COSTA L.G. **Genetic and temporal determinants of pesticide sensitivity, role of paraoxonase (PON1).** Neurotoxicology 21 (2000) 91-100.

GIACOBINI E. **New trends in cholinergic therapy for Alzheimer's disease: nicotin agonists or cholinesterase inhibitors**. Prog Brain Res 13 (1996) 655-670.

GIULIANIA F., VERNAY A., LEUBA G., SCHENKA F. **Decreased behavioral impairments in an Alzheimer mice model by interfering with TNF-alpha metabolism**. Brain Res Bull 80 (2009) 302-308.

GRIEB P. **Intracerebroventricular streptozotocin injections as a model of Alzheimer´s disease: in search of a relevant mechanism.** Mol Neurobiol 53 (2016) 1741-1752.

GRON G., BRANDENBURG I., WUNDERLICH A.P., RIEPE M.W. **Inhibition of hippocampal function in mild cognitive impairment: targeting the cholinergic hypothesis**. Neurobiol Aging 27 (2006) 78-87.

HABER M., ABDEL BAKI S.G., GRIN'KINA N.M., IRIZARRY R., ERSHOVA A., ORSI S., GRILL R.J., DASH P., BERGOLD P.J. **Minocycline plus N-acetylcysteine synergize to modulate inflammation and prevent cognitive and memory deficits in a rat model of mild traumatic brain injury.** Exp Neurol 249 (2013) 169-177.

HANS MARQUARDT, SIEGFRIED G. SCHÄFER, ROGER O. MCCLELLAN, FRANK WELSCH. **Toxicology**. Academic Press (1999) pp. 854.

HARDY J., SELKOE D.J. **The amyloid hypothesis of Alzheimer´s disease: progress and problems on the road to therapeutics.** Science 297 (2002) 353-356.

HASSELMO M.E. **The Role of Acetylcholine in Learning and Memory**. Curr Opin Neurobiol 16 (2006) 710-715.

HENNEBERG N., HOYER S. **Desensitization of the neuronal insulin receptor: a new approach in the etiopathogenesis of late-onset sporadic dementia of the Alzheimer type (SDTA)?** Arch Gerontol Geriatr 21 (1995) 63-74.

HEPPNER F.L., RANSOHOFF R.M., BECHER B. **Immune attack: the role of inflammation in Alzheimer disease.** Nature Rev Neur. 16 (2015) 358-372.

HOLDEN M., KELLY C. **Use of Cholinesterase Inhibitors in Dementia**. Adv Psych Treat 8 (2002) 89-96.

HOLDEN M., KELLY C. **Use of cholinesterase inhibitors in dementia**. Advances in Psychiatry Treatments 8 (2002) 89-96.

HOYER S., MÜLLER D., PLASCHKE K. **Desensitization of brain insulin receptor. Effect on glucose/energy and related metabolism.** J Neural Transm Suppl. 44 (1994) 259–268.

HUT R.A., VAN DER ZEE E.A. **The cholinergic system, circadian rhythmicity, and time memory**. Behav Brain Res 221 (2011) 466-480.

IBACH B., HAEN E. **Acetylcholinesterase inhibition in Alzheimer's disease.** Curr Pharm Design 10 (2004) 231-251.

IBGE. **Síntese de Indicadores Sociais Uma Análise das Condições de Vida da População Brasileira**. Instituto Brasileiro de Geografia e Estatística (2012) Rio de Janeiro.

INOUYE K., DE OLIVEIRA G.H. **Avaliação crítica do tratamento farmacológico atual para a doença de Alzheimer**. Informa 15 (2004) 80-84.

ISHIGE K., TANAKA M., ARAKAWA M., SAITO H., ITO Y. **Distinct nuclear factor-kappaB/Rel proteins have opposing modulatory effects in glutamate-induced cell death in HT22 cells.** Neurochem Int 47 (2005) 545-555.

ITO D., IMAI Y., OHSAWA K., NAKAJIMA K., FUKUUCHI Y., KOHSAKA S. **"Microglia-specific localization of a novel calcium binding protein, Iba1"**. Brain Res Mol Brain Res 57 (1998) 1-9.

JANSSEN B., VUGTS D.J., FUNKE U., MOLENAAR G.T., KRUIJER P.S., VAN BERCKEL B.N., LAMMERTSMA A.A., WINDHORST A.D. **Imaging of neuroinflammation in Alzheimer's disease, multiple sclerosis and stroke: Recent developments in positron emission tomography.** Biochim Biophys Acta 1862 (2016) 425-441.

JAVED H., KHAN M.M., KHAN A., VAIBHAV K., AHMAD A., KHUWAJA G., AHMED M.E., RAZA S.S., ASHAFAG M., TABASSUM R., SIDDIQUI M.S., EL-AQNAF O.M., SAFHI M.M., ISLAM F. **S-allyl cysteine attenuates oxidative stress associated cognitive impairment and neurodegeneration in mouse model of streptozotocin-induced experimental dementia of Alzheimer's type.** Brain Res 1389 (2011) 133-142.

JIYA S., XUEMEI F., DAPENG L., YONG D., HONGXING L. **Down-Regulation of Energy Metabolism in Alzheimer's Disease is a Protective Response of Neurons to the Microenvironment.** J Alzheimer Dis. 28 (2012) 389-402.

KEDAR N.P., COLE W.C., PRASAD K.C. **Risk factors for Alzheimer's disease: role of multiple antioxidants, non-steroidal anti-inflammatory and cholinergic agents alone or in combination in prevention and treatment**. J Am Coll Nutr 21 (2002) 506-522.

KLEIN W.L. **ADDLs & protofibrils - the missing links?** Neurobiol Aging 23 (2002) 231-233.

KRASOWSKI M.D., MCGEHEE D.S., MOSS, J. **Natural inhibitors of cholinesterases, implications for adverse drug reactions.** Can J Anaest 44 (1997) 525-534.

LI H., GUO Y., XIAO L., CHEN BO. **A fluorometric biosensor based on H2O2-sensitive nanoclusters for the detection of acetylcholine**. Biosens Bioelectron 59 (2014) 289-292.

LA DU B.N., AVIRAM M., BILLECKE S., NAVAB M., PRIMO-PARMO S., SORENSON R. C., STANDIFORD T. J**. On the physiological role(s) of the paraoxonases**. Chem Bioll Interac 119 (1999) 379-388.

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 112 (1998) 1199-1208.

LI B., STRIBLEY J.A., TICU A., XIE W., SCHOPFER L.M., HAMMOND E., BRIMIJOIN S., HINRICHS S.H., LOCKRIDGE O. **Abundant tissue butyrylcholinesterase and its possible function in the acetylcholinesterase knockout mouse**. J Neurochem 75 (2000) 1320-1331.

LISTON D.R., NIELSEN J.A., VILLALOBOS A., CHAPIN D., JONES S.B., HUBBARD S.T., SHALABY I.A., RAMIREZ A., NASON D., WHITE W.F. **Pharmacology of selective acetylcholinesterase inhibitors: implications for use in Alzheimer's disease.** Eur J Pharmacol 486 (2004) 9-17.

MANCUSO M., CALSOLARO V., ORSUCCI D., CARLESI C., CHOUB A., PIAZZA S., SICILIANO G. **Mitochondria, Cognitive Impairment, and Alzheimer's Disease.** Int J Alzheimer´s Dis 2009 (2009) 1-8.

MARK W.A., GILMORE G.C., KAYE J., MURPHY C., WINGfiELD A., BENNETT D.A., BOXER A.L., BUCHMAN A.S., CRUICKSHANKS K.J., DEVANAND D.P., DUFFY C.J., GALL C.M., GATES G.A., GRANHOLM A., HENSH T., HOLZER R., HYMAN B.T., LIN F.R., MCKEE A.C., MORRIS J.C., PETERSEN R.C., SILBERT L.C., STRUBLE R.G., TROJANOWSKI J.Q., VERGHESE J., WILSON D.A., XU S., ZHANG L.I. **At the interface of sensory and motor dysfunctions and Alzheimer's disease**. Alzheimers Dement 11 (2015) 70-98.

MARKESBERY W.R. **Oxidative Stress in Alzheimer Disease: Synergy between the Butterfield and Markesbery Laboratories.** Free Radic Biol Med 23 (1997) 134-47.

MARTINES B.M. **N-acetylcysteine elicited increase in complex I activity in synaptic mitochondria from aged mice: implications for treatment of Parkinson´s disease.** Brain Res 859 (2000) 173-175.

MARTÍNES M., HERNÁNDEZ A.I., MARTÍNES N. **N-acetylcysteine delays age-associated memory impairment in mice: role in synaptic mitochondria.** Brain Res 855 (2000) 100-106.

MCGEHEE D.S., KRASOWSKI M.D., FUNG D.L., WILSON B., GRONERT G.A., MOSS J. **Cholinesterase inhibition by potato glycoalkaloids slows mivacurium metabolism**. Anesthesiology 93 (2000) 510-519.

MCLELLAN L.I., LEWIS A.D., HALL D.J., ANSELL J.D., WOLF C.R. **Uptake and distribution of N-acetylcysteine in mice: tissue-specific effects on glutathione concentrations**. Carcinogenesis 16 (1995) 2099-2106.

MEISTER A. **Glutathione metabolism**. Methods Enzymol 251 (1995) 3-7.

MICHAELIS. **Moderno dicionário da língua portuguesa.** Ed Melhoramentos Ltda. 2007.

MILLER G.A. **The magical number seven, plus or minus two: some limits on our capacity for processing information**. Psych Review 63 (1956) 81-97.

MORRIS J.C., CYRUS P.A., ORAZEM J., MAS J., BIEBER F., RUZICKA B.B., GULANSKI B. **Metrifonate benefits cognitive, behavioral, and global function in patients with Alzheimer's disease.** Neurology 50 (1998) 1222-1230.

MORRIS J.H. **Alzheimer's disease. The neuropathology of dementia**. Cambridge: Cambridge Univ. Press, pp.70-121 (1997) London, England.

MURAMOTO O., SUGISHITA M., SUGITA H., TOYOKURA Y. **Effect of physostigmine on constructional and memory tasks in Alzheimer's disease**. Arch Neurol 36 (1979) 501-503.

MURAMOTO O., SUGISHITA M., SUGITA H., TOYOKURA Y. **Effect of physostigmine on constructional and memory tasks in Alzheimer's disease.** Arch Neurol 36 (1979) 501-503.

MURRAY-LYON I.M., EDDLESTON A.L., WILLIAMS R., BROWN M., HOGBIN B.M., BENNETT A., EDWARDS J.C., TAYLOR K.W. **Treatment of multiple-hormone-producing malignant islet-cell tumor with streptozotocin**. Lancet 2 (1968) 895-898.

NITSCH R., HOYER S. **Local action of the diabetogenic drug, streptozotocin, on glucose and energy metabolism in rat brain cortex.** Neurosci Lett 128 (1991) 199–202.

ODA Y. **Choline acetyltransferase: the structure, distribution and pathologic changes in the central nervous system**. Pathol Int 49 (1999) 921-937.

OVALDO P.A. **Tratamento da doença de Alzheimer: Avaliação crítica sobre o uso de anticolinesterásicos**. Arq. Neuropsiquiatr. 56 (1998) 688-696.

PARSONS S.M., BAHR B.A., GRACZ L.M., KAUFMAN R., KORNREICH W.D., NILSSON L., RODGERS G.A. **Acetylcholine transport: Fundamental properties and effects of pharmacologic agents**. Ann N Y Acad Sci 493 (1987) 220-233.

PERRY E.K., TOMLINSON B.E., BLESSED G. **Changes in brain cholinesterases in senile dementia of Alzheimer type**. British Medical Journal 2 (1978) 1457-1459.

PETERS B.H., LEVIN H.S. **Effects of physostigmine and lecithin on memory in Alzheimer disease**. Ann Neurol 6 (1979) 219-221.

PINTON S. **Disseleneto de p-metoxifenila atenua o prejuízo cognitivo e a injuria cerebral em um modelo da doença de Alzheimer em roedores**. Tese de doutorado - UFSM (2012) Santa Maria - RS - Brasil.

POHANKA M. **"Alzheimer´s disease and oxidative stress: a review".** Current Med Chem 21 (2013) 356-364.

QUIN H., YANG Y. **Neuron differentiation and neuritogenesis stimulated by N-acetylcysteine (NAC).** Acta Pharmacol sin 30 (2009) 907-912.

RANG H.P., DALE M.M., RITTER J.M., FLOWER R.J., HENDERSON G. **Rang & Dale´s Pharmacology**, 7th. Elsevier Inc. 2007.

RAYMOND L.A., BLACKSTONE C.D., HUGANIR R.L. **Phosphorylation of amino acid neurotransmitter receptors in synaptic plasticity**. Trends Neurosci. 16 (1993) 147-153.

RESENDE R., PEREIRA C., AGOSTINHO P., VIEIRA A.P., MALVA J.O., OLIVEIRA C.R. **Susceptibility of hippocampal neurons to A peptide toxicity is associated with perturbation of Ca2+ homeostasis**. Brain Res 1143 (2007) 11-21.

RODRIGUES F.S., SOUZA M.A., MAGNI D.V., FERREIRA A.P.O., MOTA B.C., CARDOSO A.M., PAIM M., XAVIER L.L., FERREIRA J., SCHETINGER M.R.C., DA COSTA J.C., ROYES L.F.F., FIGHERA M.R. **N-acetylcysteine prevents spatial memory impairment induced**

by chronic early postnatal glutaric acid and lipopolysaccharide in rat pups. PloS One 8 (2013) e78332.

ROGERS S.L., FARLOW M.R., DOODY R.S., MOHS R., FRIEDHOFF L.T. **A 24-week, double blind, placebo-controlled trial of donepezil in patients with Alzheimer's disease.** The Donepezil Study Group. Neurology 50 (1998) 136-144.

ROSENBERRY T.L., MALLENDER W.D., THOMAS E.J., SZEGLETES T. **Substrate binding to the peripheral site occurs on the catalytic pathway of acetylcholinesterase and leads to substrate inhibition**. pp. 189. Plenum Press (1998) New York.

SALKOVIC-PETRISIC M, KNEZOVIC A, HOYER S, RIEDERER P. **What have we learned from the streptozotocin-induced animal model of sporadic Alzheimer's disease, about the therapeutic strategies in Alzheimer's research.** J Neural Transm 120 (2013) 233–252.

SAMUNI Y., GOLDSTEIN S., DEAN O.M., BERK M. **The chemistry and biological activities of N-acetylcysteine**. Biochim Biophys Acta 1830 (2013) 4117-4129.

SÁNCHES C., DÍAZ-NILDO J., AVILA J. **Phosphorylation of microtubule-associated protein 2 (MAP2) and its relevance for the regulation of the neuronal cytoskeleton function.** Proq Neurobiol 6 (2000) 133-168.

SANSONE R.A., SANSONE L.A. **Chronic Pain Syndromes and Borderline Personality**. Innov Clin Neurosci 8 (2011) 10-14.

SAXENA G., BHARTI S., KAMAT P.K., SHARMA S., NATH C. **Melatonin alleviates memory deficits and neuronal degeneration induced by intracerebroventricular administration of streptozotocin in rats.** Pharmacol 94 (2010) 397-403.

SCOVILLE W.B., MILNER B. **Loss of Recent Memory after Bilateral Hippocampal Lesions**. J Neurol Neurosurg Psychiatry 20 (1957) 11-21.

SEN C.K. **Nutritional biochemistry of cellular glutathione.** J Nutr Biochem 8 (1997) 660-672.

SHAHRIPOUR B.R., HARRIGAN M.R., ALEXANDROV A.V. ALEXANDROV. **N-acetylcysteine (NAC) in neurological disorders: mechanisms of action and therapeutic opportunities.** Brain Behav 4 (2014) 108-122.

SHARMA B., SINGH N., SINGH M. **Modulation of celecoxib- and streptozotocin-induced experimental dementia of Alzheimer's disease by pitavastatin and donepezil**. J Psychopharmacol 22 (2008) 162-171.

SHARMA M., GUPTA Y.K. **Intracerebroventricular injection of streptozotocin in rats produces both oxidative stress in the brain and cognitive impairment**. Life Sci 68 (2001) 1021-1029.

SHARMA N., DESHMUKH R., BEDI K.L. **SP600125, a competitive inhibitor of JNK attenuates streptozotocin induced neurocognitive deficit and oxidative stress in rats.** Pharmacol Biochem Behav 96 (2010) 386-394.

SHEFFNER A.L., MEDLER E.M., BAILEY K.R., GALLO D.G., MUELLER A.J., SARETT H.P. **Metabolic studies with acetylcysteine**. Biochem Pharmacol 15 (1966) 1523-1535.

SHOHAM S., BEJAR C., KOVALEV E., SCHORER-APELBAUM D., WEINSTOCK M. **Ladostigil prevents gliosis, oxidative-nitrative stress and memory deficits induced by intracerebroventricular injection of streptozotocin in rats**. Neuropharmacology 52 (2007) 836-843.

SIEGEL G.J., AGRANOFF B.W., ALBERS R.W., LIPPINCOTT-RAVEN. **Basic Neurochemistry: Molecular, Cellular and Medical Aspects**. 6th ed (1999) Philadelphia.

SILVER A. **The Biology of Cholinesterases.** Frontiers of Biology, 36. (1974) Amsterdam. Holland.

SINKO G., CALIĆ M., BOSAK A., KOVARIK Z. **Limitation of the Ellman method: Cholinesterase activity measurement in the presence of oximes**. Anal Biochem 370 (2207) 223-227.

SMITH C.M., SWASH M. **Possible biochemical basis of memory disorder in Alzheimer disease.** Ann Neurol 3 (1978) 471-473.

SPERLING G. **A Model for Visual Memory Tasks.** Hum Fact 5 (1963) 19-31.

STIX G. **Alzheimer´s: forestalling the darknes**. Sci Am 302 (2010) 50-57.

STRELOW J., DEWE W., IVERSEN P.W., BROOKS H.B., RADDING J.A, MCGEE J. WEIDNER J. **Mechanism of Action Assays for Enzymes**. Assay Guid Amn (2012) Bathesda (MD): Eli Lilly & Company.

THAKURTA I.G., BANERJEE P., BAGH M.B., GHOSH A., SAHOO A., CHATTOPADHYAY S., CHAKRABARTI S. **Combination of N-acetylcysteine, α-lipoic acid and α-tocopherol substantially prevents the brain synaptosomal alterations and memory and learning deficits of aged rats.** Exp Gerontol 50 (2014) 19-25.

TUCEK S. **The synthesis of acetylcholine: Twenty years of progress.** Prog Brain Res 84 (1990) 467-477.

VERKHRATSKY A., OLABARRIA M., NORISTANI H., YEH C., RODRIGUEZ J.J. **Astrocytes in Alzheimer's Disease.** NeuroTherapeutics. 7 (2010) 399-412.

WATKINS P.B., ZIMMERMAN H.J., KNAPP M.J., GRACON S.I., LEWIS K.W. **Hepatotoxic effects of tacrina administration in patients with Alzheimer's disease.** JAMA 271 (1994) 992-998.

WHO. Dementia - **A public health priority.** World Health Organization (2012). http://www.alz.co.uk./WHO-dementia-report.

WOLTJER R.L., MILATOVIC D. **Toxicology of Organophosphate & Carbamate Compounds.** 3 (2006) University of Washington, Seattle, Washington.

XIE W., STRIBLEY J.A., CHATONNET A., WILDER P.J., RIZZINO A., MCCOMB R.D., TAYLOR P., HINRICHS, S.H., LOCKRIDGE O. **Postnatal developmental delay and supersensitivity to organophosphate in gene targeted mice lacking acetylcholinesterase**. J Pharmacol Exper Therapeut 293 (2000) 896-902.

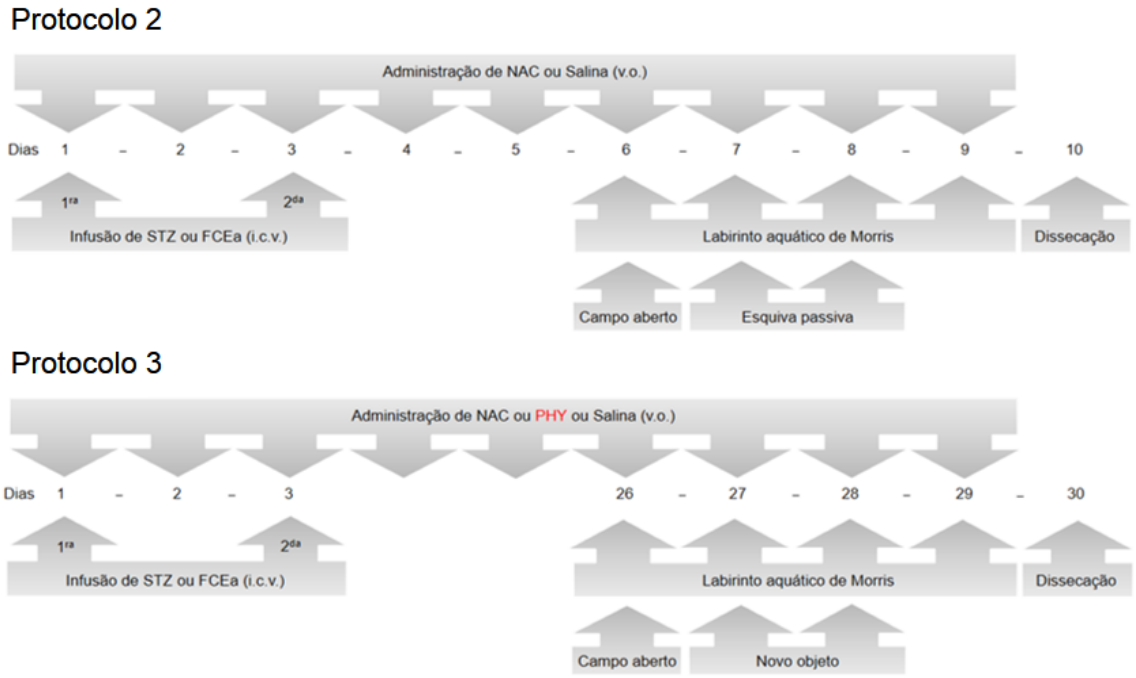
XU J.H., HU H.T., LIU Y., QIAN Y.H LIU Z.H., TAN Q.R., ZHANG Z.J. **Neuroprotective effects of ebselen are associated with the regulation of Bcl-2 and Bax proteins in cultured mouse cortical neurons.** Neurosci Lett 399 (2006) 210-214.

ZAJICEK J. **Studies on the histogenesis of blood platelets and megakaryocytes**. Acta Physiol Scand 40 (1957) 1-32.

APÊNDICES

Apêndice 1

Protocolos de administração *in vivo*



Apêndice 2

Effects of NAC administration on myeloperoxidase (MPO) activity in mice that received i.c.v. injection of STZ.

	MPO (optic density/h/mg protein)		
	Cerebral cortex	**Hippocampus**	**Striatum**
Sham	9.5 ± 1.0	6.0 ± 0.5	6.4 ± 0.7
NAC	6.8 ± 0.9	6.2 ± 0.8	5.2 ± 0.3
STZ	8.5 ± 0.6	5.7 ± 0.5	6.4 ± 0.7
NAC+STZ	8.5 ± 0.9	6.1 ± 0.5	5.3 ± 0.6

Data are reported as means ± S.E.M for n = 6 to 8 animals per group. Statistical analysis was performed by two-way ANOVA followed by Duncan's test.

Apêndice 3

Effects of NAC administration on ABTS$^{\bullet}$ scavenger capacity in mice that received i.c.v. injection of STZ.

ABTS$^{\bullet}$ (µmol of VitC/mg tissue)			
	Cerebral cortex	**Hippocampus**	**Striatum**
Sham	155.7 ± 13.4	224.2 ± 9.2	256.0 ± 11.8
NAC	156.6 ± 14.3	210.4 ± 12.3	214.1 ± 10.7
STZ	172.6 ± 11.3	200.4 ± 16.1	241.1 ± 21.2
NAC+STZ	209.4 ± 14.2	244.5 ± 11.8	233.1 ± 16.6

Data are reported as means ± S.E.M for n = 6 to 8 animals per group. Statistical analysis was performed by two-way ANOVA followed by Duncan's test.

Apêndice 4

Effects of NAC administration on TSH and NPSH levels in the brain of mice that received i.c.v. injection of STZ.

TSH (µmol SH/mg tissue)			
	Cerebral cortex	**Hippocampus**	**Striatum**
Sham	5.6 ± 0.3	5.7 ± 0.3	5.1 ± 0.1
NAC	5.4 ± 0.3	5.4 ± 0.1	4.9 ± 0.1
STZ	5.3 ± 0.1	5.6 ± 0.3	5.1 ± 0.1
NAC+STZ	5.3 ± 0.1	5.1 ± 0.1	5.3 ± 0.2

NPSH (µmol SH/mg tissue)			
	Cerebral cortex	**Hippocampus**	**Striatum**
Sham	2.8 ± 0.0	3.3 ± 0.0	3.4 ± 0.0
NAC	2.7 ± 0.0	3.2 ± 0.0	3.4 ± 0.1
STZ	2.7 ± 0.0	3.2 ± 0.1	3.4 ± 0.1
NAC+STZ	2.7 ± 0.0	3.1 ± 0.1	3.3 ± 0.0

Data are reported as means ± S.E.M for n = 6 to 8 animals per group. Statistical analysis was performed by two-way ANOVA followed by Duncan's test.