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EFEITOS DO PRÉ-TRATAMENTO COM MEMANTINA EM UM MODELO DE NEURODEGENERAÇÃO INDUZIDO PELA ADMINISTRAÇÃO INTRAHIPOCAMPAL DE ÁCIDO OCADÁICO EM RATOS: UMA AVALIAÇÃO COMPORTAMENTAL E NEUROQUÍMICA

Eduardo Rigon Zimmer

Porto Alegre, julho de 2011

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SUMÁRIO

Parte I

Resumo

A Doença de Alzheimer (DA) é uma doença cerebral progressiva que resulta em prejuízos na memória e disfunção cognitiva global. Entre as principais características neuropatológicas associadas a DA estão à presença de placas senis, emaranhados neurofibrilares e a hiperfosforilação da proteína Tau. A hiperativação do sistema glutamatérgico tem sido implicada na fisiopatologia da DA. O excesso de glutamato na fenda sináptica causa hiperativação do seu receptor ionótropico N-metill-D-aspartato (NMDA) o que favorece o aumento do influxo de cálcio e morte neuronal. A administração intracerebral de ácido ocadáico (AO) causa alterações morfológicas e funcionais similares à DA. O AO promove a inibição da proteína fosfatase 2A (PP2A) favorecendo as atividades cinásicas de proteínas como a cinase dependente de ciclina 5 (Cdk5). A memantina (MN) é uma das principais drogas utilizadas no tratamento da DA e o seu mecanismo de ação envolve um antagonismo não competitivo de baixa afinidade pela subunidade NR2B do receptor NMDA. Neste trabalho, foram avaliados efeitos do pré-tratamento com MN em um modelo semelhante a DA induzido pela administração intrahipocampal de AO em ratos. O pré-tratamento com MN preveniu o déficit na memória especial causado pela infusão intrahipocampal de AO. Os mecanismos envolvidos nestes efeitos neuroprotetores envolvem a prevenção do aumento de glutamato no liquido cefalorraquidiano, juntamente com a regulação da expressão de Cdk5 e em conseqüência a prevenção do aumento da fosforilação de Tau. Desta maneira, a MN pode ser um alvo terapêutico para prevenir as alterações comportamentais e neuroquímicas em um modelo similar a DA induzido pelo AO.

Palavras-Chave: Memantina; ácido ocadáico; doenças neurodegenerativas; glutamato; Cdk5, memória e aprendizado.

Abstract

Alzheimer's disease (AD) is a progressive brain disease that causes memory loss and global cognitive dysfunction. The neuropathological alterations associated with AD include senile plaques, neurofibrillary tangles and Tau protein hyperphosphorylation. The glutamatergic system is implicated in the pathophysiology of AD. Indeed, the excessive glutamate levels in the synaptic cleft may cause hyperactivation of glutamate ionotropic N-metill-D-aspartate (NMDA), which favors increase calcium influx and neuronal death. The intracerebral administration of okadaic acid (OA) causes morphological and functional alterations similar to AD. The OA inhibits the protein phosphatase 2A (PP2A) thus overstimulating the kinases activities. Memantine (MN) is a drug currently used in the treatment of AD, which mechanism involves a non-competitive low affinity antagonism for NR2B subunit of NMDA receptors. In this work we evaluate the effects of pretreatment with MN in an AD-like model in rats induced by intrahippocampal administration of OA. The pretreatment with MN could prevent the spatial memory deficits caused by OA intrahipocampal administration in rats. The mechanisms underlying this neuroprotective effects involves the prevention of the increase in brain glutamate levels along with regulation of Cdk5 and, in consequence, downstream phosphorylation of Tau (ser199/202) protein. To conclude, MN has potential therapeutic role in preventing behavioral and neurochemical alterations caused by an AD like model induced by OA.

Keywords: Memantine; okadaic acid, neurodegenerative diseases, glutamate, Cdk5, learning and memory.

Lista de Abreviaturas

AO – Ácido Ocadáico

CDK5 – Cinase dependente de ciclina 5

DA – Doença de Alzheimer

LCR – Líquido Cefalorraquidiano

MN – Memantina

NMDA – N-metil-D-aspartato

NMDAr – receptor N-metil-D-aspartato

PP2A – proteína fosfatase 2A

SNC – Sistema nervoso Central

Apresentação

Esta dissertação está constituída por introdução, artigo científico em preparação, discussão, conclusão e referências bibliográficas.

1. Introdução

1.1 Doença de Alzheimer e o seu impacto na sociedade

O aumento da expectativa de vida da população e o estilo de vida ocidental têm contribuído para a prevalência de doenças neurodegenerativas, como a doença de Alzheimer (DA) e outras demências (Enna and Coyle, 1998; Ho et al., 2010). A DA causa uma diminuição na qualidade de vida dos pacientes e tem um elevado custo social e econômico para as famílias e para os sistemas públicos de saúde (Dartigues et al., 2002). Esta doença tornou-se uma epidemia e um problema de saúde pública. Existem mais de 35 milhões de portadores da DA (Ferri et al., 2005; Irvine et al., 2008; Brambilla et al., 2011), e em sua maioria com mais de 60 anos de idade (Citron, 2004; Querfurth and LaFerla, 2010).

1.2 Características fisiopatológicas da doença de Alzheimer

A DA é uma doença cerebral progressiva que resulta em prejuízos na memória, raciocínio, aprendizado e alterações de personalidade, e em geral causa uma disfunção cognitiva global (Katzman, 1986; Delbeuck et al., 2003; Ho et al., 2010). Entre as principais características neuropatológicas associadas a DA estão à presença de placas senis, emaranhados neurofibrilares e a hiperfosforilação da proteína Tau em regiões do cérebro responsáveis por cognição e memória. Estas alterações estão demonstradas na figura 1 (McGeer and McGeer, 2007; Citron, 2010; Ittner and Gotz, 2011).

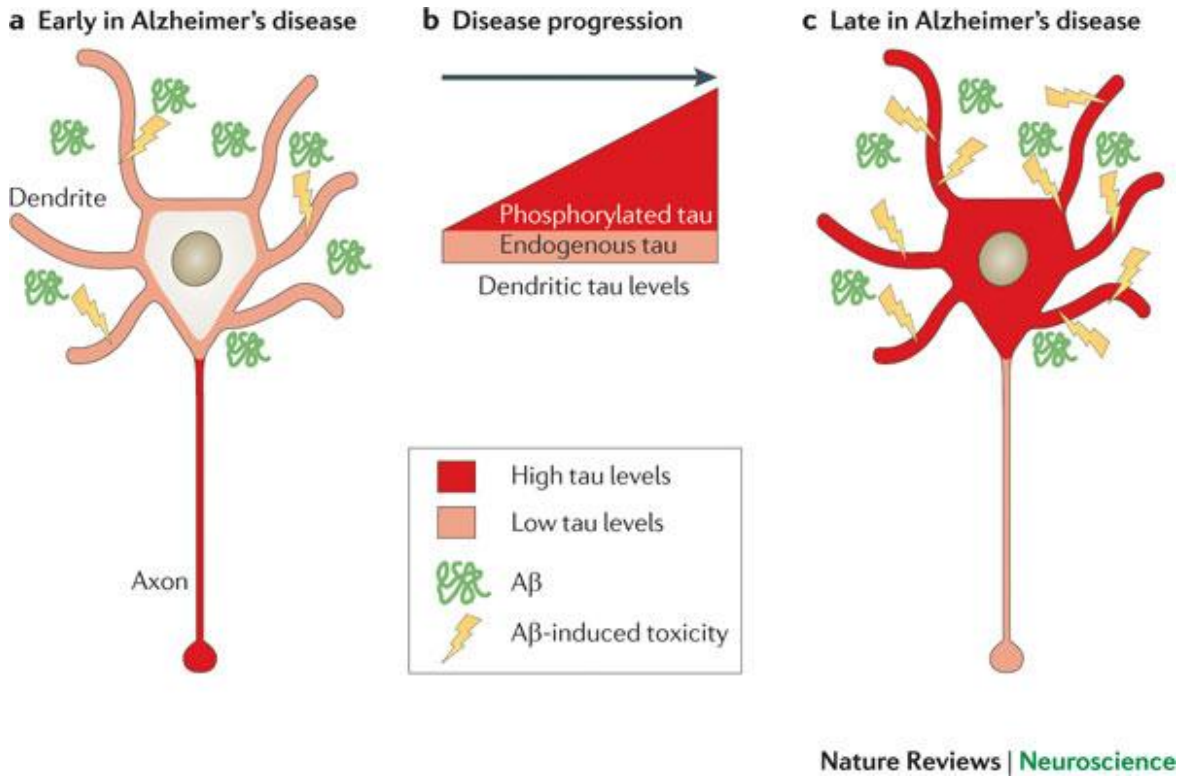

Figura 1. Relação entre as placas senis e a fosforilação da proteína Tau na progressão da doença de Alzheimer (adaptado de Ittner and Gotz, 2011).

1.3 A Evolução das investigações em sistemas de neurotransmissão na doença de Alzheimer

As investigações dos mecanismos fisiopatológicos da DA foram inicialmente direcionadas para o entendimento das alterações funcionais do sistema de neurotransmissão colinérgico, porém o arsenal terapêutico disponível no mercado com essa finalidade não consegue evitar a progressão da doença (Nicolakakis and Hamel, 2011). Recentemente, a participação de outros sistemas de neurotransmissão, em especial o glutamatérgico, estão sendo investigados na tentativa de se encontrar novos alvos terapêuticos. (Sonkusare et al., 2005).

1.4 O envolvimento do sistema glutamatérgico em doenças neurodegenerativas

O Glutamato é reconhecido como o principal neurotransmissor excitatório do sistema nervoso central (SNC) e está envolvido na manutenção das funções normais do cérebro, como desenvolvimento, indução da sinapse, plasticidade, migração celular, diferenciação e morte neuronal (Danbolt, 2001). Muitos estudos mostram que o glutamato está intrinsecamente ligado com o bom funcionamento da memória e cognição assim como o desenvolvimento normal do cérebro (Collingridge and Lester, 1989; Headley and Grillner, 1990; Danbolt, 2001; Filali et al., 2011). Porém a hiperestimulação do sistema glutamatérgico pode exercer um efeito excitotóxico e causar morte de células neurais e aumento da reatividade astrocitária (Maragakis and Rothstein, 2004; Graeber and Streit, 2010). Uma vez liberado pelas vesículas sinápticas, o glutamato age na fenda sináptica pela sua ligação a receptores pós-sinápticos metabotrópicos (mGluRr1-8) e ionotrópicos (AMPAr, KAr, NMDAr) (Hollmann and Heinemann, 1994). Neste contexto, a captação de glutamato pelos astrócitos constitui uma importante linha de defesa para evitar a excitotoxicidade (Leke et al., 2006; Almeida et al., 2010). O sistema glutamatérgico parece estar hiperestimulado em doenças agudas e crônicas que cursam com o comprometimento do SNC incluindo a DA (Dingledine et al., 1999). Nesse sentido, o receptor ionótropico N-metill-D-aspartato (NMDA) tem sido o mais explorado nos últimos anos. Este receptor é um canal iônico heterodimérico formado por subunidades Nr1, Nr2 (a/b/c/d) e Nr3 (a/b), sendo que essas desempenham diferentes papéis regulatórios na neurotransmissão glutamatérgica (Schoepfer et al., 1994; Cull-Candy et al., 2001).

1.5 Implicações da via de sinalização da cinase dependente de ciclina 5 (Cdk5) em doenças neurodegenerativas

A busca por vias de sinalização celulares envolvidas em processos de sobrevivência e neurodegeneração na DA tem sido alvo de grande interesse. Recentemente, a hiperatividade da cinase dependente de ciclina 5 (Cdk5) foi associada a processos de neurodegeneração cerebral (Wang et al., 2003; Crews and Masliah, 2010). Estudos mostram que os níveis de Cdk5 estão aumentados em pacientes com DA (Ahlijanian et al., 2000). Entretanto, a Cdk5 tem um importante papel na plasticidade e desenvolvimento neuronal. Existem dois ativadores fisiológicos da Cdk5: p35 e p39. Em situações onde existem altas concentrações de cálcio o p35 é clivado pela calpaína em p25. A p25 se liga a Cdk5 o que promove a fosforilação aberrante da Tau, como demonstrado na figura 2, e das subunidades regulatórias do receptor NMDA (Patrick et al., 1999; Dhavan and Tsai, 2001; Zhang et al., 2008; Crews and Masliah, 2010). Desta maneira, esta via de sinalização parece estar fortemente implicada no processo neurodegenerativo da DA.

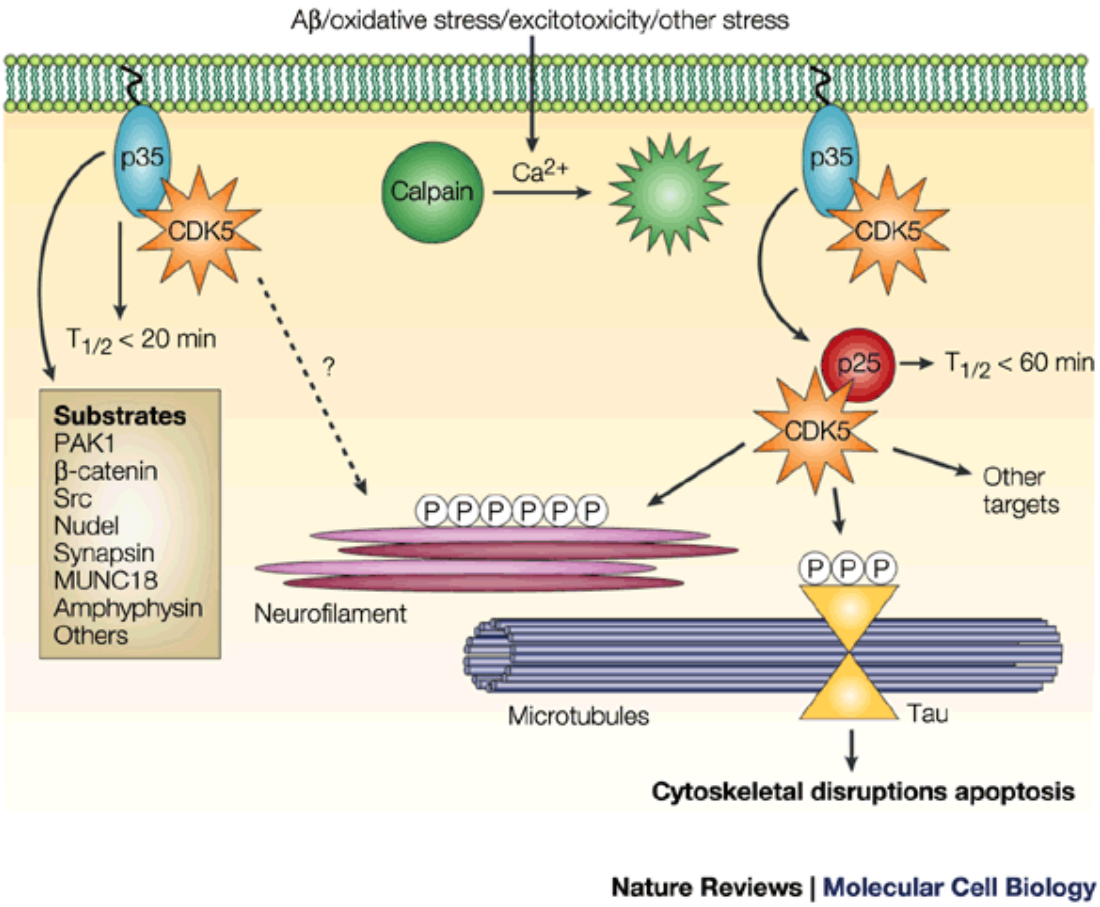

Figura 2. Mecanismo de fosforilação da Tau via Cdk5. Esta figura nos mostra, o mecanismo de ação fisiológico da Cdk5 ativado pela p35 e o mecanismo de ação patológico onde Cdk5 é ativado por p25 e fosforila a proteína Tau (adaptado de Dhavan and Tsai, 2001).

1.4 A Utilização da memantina no tratamento da doença de Alzheimer

A memantina (MN) é uma das principais drogas utilizadas atualmente no tratamento da DA, pois ela produz uma melhora global nos aspectos comportamentais e cognitivos dos pacientes retardando a progressão da doença (Winblad and Poritis, 1999; Reisberg et al., 2003; Tariot et al., 2004; Peskind et al., 2006; van Marum, 2009). O mecanismo de ação da MN envolve um antagonismo não competitivo de baixa afinidade na subunidade NR2B do

receptor NMDA, como demonstrado na figura 3. Isso leva a um bloqueio parcial do influxo excessivo de cálcio decorrente do aumento de glutamato na fenda sináptica, mas é capaz de manter a atividade do receptor em níveis fisiológicos (Johnson and Kotermanski, 2006; Lipton, 2007).

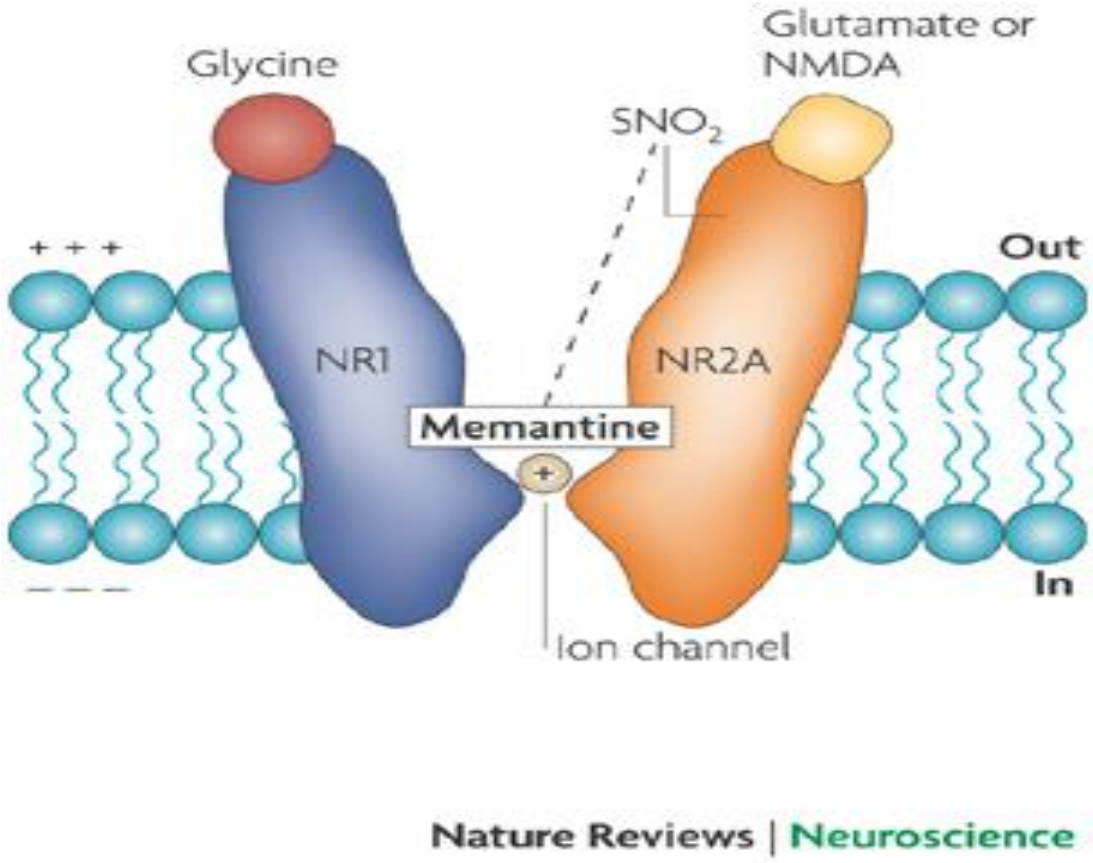

Figura 3. Mecanismo de ação memantina no receptor NMDA causando o bloqueio parcial do influxo de cálcio (adaptado de Lipton, 2007).

Sabe-se que a MN reverte a hiperfosforilação da Tau, em modelos experimentais da DA (Li et al., 2004). Recentemente também foi demonstrado que o pré-tratamento com MN tem uma ação neuroprotetora em ratos submetidos à isquemia (Babu and Ramanathan, 2009). No entanto há poucos estudos utilizando o pré-tratmento com MN para avaliar seus possíveis efeitos neuroprotetores. Sendo assim, as potencialidades e as possíveis ações preventivas da MN requerem mais investigações.

1.5 O Impacto da administração de ácido ocadáico no Sistema nervoso central

A administração intracerebral de ácido ocadáico (AO), uma potente neurotoxina, tem sido muito utilizada como um modelo de neurodegeneração, pois causa alterações morfológicas e funcionais similares à DA (Arendt et al., 1998; Arias et al., 1998; Arias et al., 2002; Zhang et al., 2008). O AO promove a inibição da proteína fosfatase 2A (PP2A) e o consequente favorecimento das atividades cinásicas, levando assim a um estado celular hiperfosforilado. De acordo com isso, estudos demonstram que a inibição desta fosfatase causa uma fosforilação aberrante da proteína Tau e da subunidade regulatória NR2B do receptor NMDA, contribuindo assim para o processo de degeneração das células neuronais e ativação de células gliais (Arendt et al., 1995; Arendt et al., 1998; Bennecib et al., 2000; Block and Hong, 2007; Zhang et al., 2008).

1.6 A busca por um diagnóstico clínico da doença de Alzheimer

O diagnóstico precoce da DA poderia atenuar ou até mesmo impedir a progressão da doença. É importante ressaltar que diferentes grupos têm conduzido estudos clínicos na tentativa de identificar possíveis marcadores genéticos, bioquímicos ou novas ferramentas capazes de detectar precocemente indivíduos que apresentem maior risco de desenvolver a DA e indivíduos em estágios iniciais da doença (Tan et al., 2007; Reitz and Mayeux, 2009; van Exel et al., 2009; Vialatte et al., 2011). Além disso, estudar alterações cerebrais envolvidas nas doenças neurodegenerativas, especialmente *in vivo*, tem sido um permanente estímulo para a busca de marcadores periféricos que possam refletir como o cérebro reage aos mais

variados estímulos (Busnello et al., 2006). Atualmente não existe um teste diagnóstico definitivo para a DA, dessa maneira, o diagnóstico clínico é baseado na sintomatologia, sendo que a confirmação só é feita no post-mortem (Qin et al., 2009).

Embora estratégias farmacológicas preventivas em doenças neurodegenerativas possam ser uma perspectiva relevante, a investigação do potencial terapêutico do pré-tratamento com MN tem sido pouco explorada. Uma vez que a utilização da MN como estratégia farmacológica preventiva evite ou minimize os danos comportamentais e neuroquímicos causados pelo AO em ratos**,** novos estudos poderão ser direcionados para o entendimento dos mecanismos celulares e moleculares associados aos efeitos neuroprotetores.

2. Objetivos

2.1. Objetivo Geral

Investigar os efeitos farmacológicos, comportamentais e neuroquímicos do pré-tratamento com MN como uma intervenção neuroprotetora em um modelo similar a DA induzido pela administração intrahipocampal de AO.

2.2. Objetivos específicos

- Investigar os efeitos do AO e da MN comportamentais na memória, aprendizado e locomoção espontânea;
- Avaliar os efeitos do AO e da MN na sinalização glutamatérgica no hipocampo e nos níveis de glutamato em liquido cefalorraquidiano de ratos;

- Avaliar os efeitos do AO e da MN no imunoconteúdo da subunidade NR2B do receptor NMDA
- Investigar a participação da via de sinalização da Cdk5 no modelo de neurodegeneração induzido pelo AO assim como os níveis de fosforilação da proteína Tau
- Avaliar possíveis associações entre o imunoconteúdo de Cdk5, NR2B, Tau após a administração do AO e os parâmetros comportamentais.

Parte II

Capítulo I - Artigo em preparação

Pretreatment with memantine prevents Alzheimer-like alterations induced by intrahippocampal okadaic acid administration in rats

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Abstract

Cerebral okadaic acid (OA) administration is proposed as a model of neurodegeneration similar to Alzheimer disease. The role of ciclin dependent kinase 5 (Cdk5), NMDA receptor and Tau proteins in the processes of neurodegeneration induced by OA still remains unclear. We investigate the effects of pretreatment with memantine (MN) in a neurodegenerative model induced by intrahipocampal okadaic acid (OA) administration. Male Wistar rats 4-5 months old (n=55) were treated intraperitoneally during 3 days with MN (20mg/kg) and afterwards, they received an intrahipocampal infusion of OA (100 ng) at right hemisphere. Animals were divided in: control (CO), MN, OA and OA/ MN groups. The spontaneous locomotion in the open field and spatial memory performance in Morris water maze were assessed. Additionally, we measured cerebrospinal fluid (CSF) glutamate levels and the immunocontent of Cdk5, p39, Calpain-2, NR2B and Tau in hippocampus. Spontaneous locomotion was not different among groups. The OA infusion causes a significant decrease in the spatial memory performance in Morris water maze. Further, OA increases CSF glutamate levels and the immunocontent of Cdk5 and phospho Tau in hippocampus. Conversely, pretreatment with MN prevented: i) spatial memory deficits, ii) augment of CSF glutamate levels and iii) the increase in the immunocontent of Cdk5 and phosphorylated Tau caused by OA. Thus, pretreatment with MN seems to be effective in prevent spatial memory deficits in an AD like model induced by OA. The decline in CSF glutamate levels along with reduced expression of Cdk5 and downstream Tau hyperphosphorylation may participate in the mechanisms underlying the neuroprotective effects of MN.

Keywords: Memantine, okadaic acid, neurodegenerative diseases, glutamate, learning and memory.

1. Introduction

Alzheimer's disease (AD) is an aging-associate neurodegenerative disease that causes important brain structural and neurochemical alterations associated with progressive deterioration of cognitive function (Katzman, 1986; Delbeuck et al., 2003; Ho et al., 2010). The pathological characterization of AD comprise the accumulation of senile plaques (deposits of amyloid-β) and neurofibrillary tangles (NTFs) formed by accumulation of abnormal filaments of Tau protein in brain regions that serve memory and cognition (McGeer and McGeer, 2007; Citron, 2010).

For many years the acetyl cholinesterase inhibitors were the first choice drugs for the treatment of AD, but recently the glutamatergic system also shows to be a possible target for new drugs therapy (Sonkusare et al., 2005) (Nicolakakis and Hamel, 2011). Glutamate is considered the major excitatory neurotransmitter in brain and plays fundamental roles in the neurodevelopment, neuronal survival and learning and memory processes through its interactions with ionotropic (AMPAr, KAr and NMDAr) and metabotropic receptors (mGluRr) (Headley and Grillner, 1990; Hollmann and Heinemann, 1994; Danbolt, 2001) . However, increased amounts of glutamate in the synaptic cleft may causes receptors hyperactivation and neuronal death by excitotoxicity (Maragakis and Rothstein, 2004). In this context, N-methyl-D-aspartate receptor (NMDAr) exerts a major role in a variety of neurodegenerative disorders including AD (Dingledine et al., 1999; Filali et al., 2011). The NMDAr is a heterodimeric calcium ion channel composed by essential (NR1) and regulatory subunits (NR2A/B/C/D and NR3A/B). The excessive calcium influx through the ion channel leads to activates signaling pathways involved in neurodegeneration (Danbolt, 2001). The brain cyclin dependent kinase (Cdk5) activity is

physiologically regulated by p35 or p39 proteins. However, under high intracellular calcium concentrations, p35 is cleaved by calpain into p25 originating the complex Cdk5/p25 which causes downstream aberrant phosphorylation of tau and NMDA NR2A/B subunits. Several line of evidences have associated Cdk5 pathway with AD pathogenesis (Patrick et al., 1999; Zhang et al., 2008; Crews and Masliah, 2010).

Memantine (MN), a non-competitive antagonist of low affinity for NR2B subunit has been used in the treatment of AD to delay neurodegenerative processes and improve cognitive function (Winblad and Poritis, 1999; Reisberg et al., 2003; Tariot et al., 2004; Peskind et al., 2006; van Marum, 2009). The mechanism of action involves the blockade of excessive influx of calcium through the NMDA receptor (Johnson and Kotermanski, 2006). Recently, a pretreatment with MN was able to reduce the brain damage caused in an ischemic insult (Babu and Ramanathan, 2009). This evidence gives support to the conjecture that MN may have a role as preventive pharmacological approach addressed to minimize the harmful effects of glutamate excitotoxicity in experimental models of neurological diseases. In this context, few works have explored the relevance of pretreatment of MN in AD models. The intracerebral administration of okadaic acid (OA) causes a selective inhibition of protein phosphatase 2A (PP2A) and induces AD like alterations including hyperphosphorylation of Tau and NR2B subunit, glial damage and cognitive deficits (Arendt et al., 1998; Bennecib et al., 2000; Arias et al., 2002; Zhang et al., 2008).

The main goal of this work was to investigate in rats whether pretreatment with memantine can prevent AD like alterations induced by OA.

With this aim, we assessed spontaneous locomotion, spatial memory performance and the expression of Cdk5 and downstream Tau and NR2B subunit proteins in rats pretreated with MN in a AD like model induced by OA.

2. Methods

2.1 Animals

Adult Male Wistar rats weighing between 400 and 500 g, 4-5 months old were obtained from Stated Foundation for Health Science Research (FEPPS, Porto Alegre/RS, Brazil). Animals were allocated into a room with controlled temperature (22ºC), under a 12-h light/12-h dark cycle and all of them having free access to food and water. Rats were divided into four groups (n=14-17, per group): control (CO), memantine (MN), okadaic acid (OA) and okadaic acid/memantine (OA/MN). To avoid social isolation we kept 4 animals for cage (Leasure and Decker, 2009). All behavioral tests were performed between 9:00 a.m. and 5:00 p.m. All experiments were in agreement with Committee on Care and Use of Experimental Animal Resources, UFRGS, Brazil.

2.2 Drugs

We used memantine (Ref.M-9292, Sigma, USA) and Okadaic acid (Ref. O8010, Sigma, EUA) dissolved in a saline (0.9 g%) to a concentration of 5 mg/ml and 50 ng/uL, respectively. All other reagents used are from analytical grade.

2.3 Treatment and Surgical procedure

The animals received a single intraperitoneal (i.p) injection of memantine (20mg/kg) or saline (NaCl 0,9%) during 3 consecutive days. In the third day animals were anesthetized by an i.p injection of ketamine (100 mg/kg body weight) and xylazine (10 mg/kg body weight). An infusion of okadaic acid (100ng) was made into the right hippocampus in the CA1 region following coordinates, with reference to bregma : A 3.6, L 2.0, and V 2.4 (Paxinos and Watson, 1986; Arias et al., 1998). In the third day post surgical procedure, rat already presented normal food intake, water consumption and spontaneous locomotion and were considered able for *in vivo* experiments.

2.4 Open field task

The open field test represents a widely used model for the evaluation of spontaneous locomotory activity. The apparatus was made of circular black-painted box (60 cm diameter x 50 cm height). The experiments were conducted in a sound-attenuated room under low-intensity light (12 lx). The rats (n=10, per group), one by one, were placed in the center of the arena and locomotor activity, anxiety-like behavior and exploration was recorded with a video camera during 10 min. The analysis was performed using a computer-operated tracking system (Any-maze, Stoelting, Woods Dale, IL).

2.5 Morris water maze task

The apparatus was a black circular pool (190 cm diameter x 70 cm height) with water temperature at 21 ± 1 °C. Rats (n=12, per group) were trained daily in a 4-trial water maze task for 4 consecutive days, with each trial lasting up to 60 s with 20 s of rest in a hidden black platform. During training,

the animals learned to escape from water by finding a hidden black platform submerged about 2 cm below the water surface in a fixed location. If animal failed to find the platform in 60 s, it was manually gently placed on the platform and allowed to rest for 20s. Immediately after each daily training session they returned to their home cages. The maze was located in a well-lit white room with several visual stimuli hanging on the walls, to provide spatial cues. Escape latency to find the platform during each trial was measured as an indicator of learning. A probe test without the platform was performed in the fifth day. The time spent in target quadrant was measured as an indicator of memory retention.

2.6 Immunohistochemistry

After 24 h of OA infusion the animals (n=2, per group) were killed and the brains were post-fixed in 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4, then cryoprotected in a 30% sucrose solution at 4 °C. Coronal sections (50 μm) were obtained using a Vibratome (Leica, Germany). Next, the slices were stained with DAPI (0.0001%; Milipore) to stain the nuclei. Sections were analyzed and photographed with a confocal microscope (Olympus, Japan).

2.7 Cerebrospinal fluid (CSF) sampling

The rats were anesthetized with of ketamine (100 mg/kg body weight) and xylazine (10 mg/kg body weight), and placed in a stereotaxic apparatus. The CSF was collected (40 to 80 mL) by direct puncture of the cisterna magna with an insulin syringe (27 gauge×1/2-inch length). Individual samples with

visible blood contamination were discarded. All samples were centrifuged at 10.000 g at 4 °C for 10 min and after that stored in single tubes at - 80 °C.

2.8 High-performance liquid chromatography (HPLC) procedure

HPLC was performed with aliquots obtained from the CSF cell-free supernatants to measure glutamate levels. The measurement was done as described previously (Schmidt et al., 2009). Analyses were performed with the Shimadzu Class-VP chromatography system, consisting of a quaternary gradient pump with vacuum degassing and piston desalting modules, Shimadzu SIL-10AF auto injector valve with 50 mL loop and a UV detector (Shimadzu, Kyoto, Japan). Separations were achieved on a Supelco 250 mm×4.6 mm, 5 µm particle size column (Supelco, St Louis, MO, USA). The mobile phase flowed at a rate of 1.2 mL/min and the column temperature was 24 °C. Buffer composition remained unchanged (A: 150 mmol/L phosphate buffer, pH 6.0, containing 150 mmol/L potassium chloride; B: 15% acetonitrile in buffer A). The gradient profile was modified to the following content of buffer B in the mobile phase: 0% at 0.00 min, 2% at 0.05 min, 7% at 2.45 min, 50% at 10.00 min, 100% at 11.00 min, and 0% at 12.40 min. Samples of 10 µl were injected into the injection valve loop. Absorbance was read at 360 and 455 nm (emission and excitation, respectively). CSF concentrations of glutamate are expressed as mean±SEM in micromoles.

2.9 Western blotting

For Western blot analysis, hippocampal homogenates were prepared in PIK buffer (1 % NP-40, 150 mM NaCl, 20 mM Tris, pH 7.4, 10% glycerol, 1 mM CaCl2, 1 mM MgCl2, 400 µM sodium vanadate, 0.2 mM PMSF, 1 µg/ml

leupeptin, 1 µg/ml aprotinin, and 0.1 % phosphatase inhibitor cocktails I and II of Sigma-Aldrich) and centrifuged. Supernatants were collected and total protein was measured by the method of Peterson (Peterson, 1977). Samples containing 40 µg of protein from homogenate of hippocampus were separated by electrophoresis on a polyacrylamide gel and electrotransfered to PVDF membranes. Non-specific binding sites were blocked with in Tween–Tris buffered saline (TTBS, 100 mM Tris–HCl, pH 7.5) containing 5% albumin for 2 h and then incubated overnight at 4 ^{o}C with monoclonal and polyclonal antibodies against Cdk5 (Cell Signaling Technology, 1:1000), p39 (Cell Signaling Technology, 1:1000, Tau$^{ser199/202}$ (Invitrogen, 1:1000), Tau (Santa Cruz, 1:200), NR2B (Cell Signaling Technology, 1:1000) , NR2B^{tyr1070} (1:1000 Cell Signaling Technology) and actin (Sigma, 1:5000). After rinsing three times for 10 min each with TTBS, membranes were incubated with secondary antibodies (1:3000 dilution, anti-rabbit, Cell Signaling Technology; 1:5000 dilution anti-mouse, Santa Cruz Technology) during 2 h at room temperature. After rinsing four times for 10 min each with TTBS, membranes were incubated with peroxidase-conjugated for 5 min at room temperature, then displayed on autoradiographic film by chemiluminescence. The films were scanned and band intensity was analyzed using ImageJ software (developed at the U.S. National Institutes of Health, and available on the Internet at http://rsb.info.nih.gov/nih-image).

2.10 Statistical analysis

Results are presented as means ±SEM. The data from the water maze task were analyzed using repeated-measures analysis of variance (ANOVA), followed by Tukey's post-hoc test. Differences between all groups were

analyzed by using analysis of variance (ANOVA) with a Tukey's post-hoc test. Differences between groups were considered statistically significant if $P < 0.05$.

3. Results

3.1 Representative image of OA infusion

After 24 h of intrahippocampal infusion of OA there is visible signs of damage on the CA1 sub region at the local of infusion (Fig.1A).

3.2 Open field Task

The administration of MN and OA did not cause significant changes in the exploratory and locomotor activity between groups (Fig.2A) in the open field session. There are no statistical significant differences in the total distance travelled in 10 min and in the mean speed (Fig.2B,2C), suggesting no locomotor deficits. Further, there are no statistical differences in the time and total distance travelled in central zone (Fig.2D, 2E).

3.3 Morris water Maze Task

During the acquisition sessions (the first 4 days), rats from the CO, MN and OA/MN groups showed improvement in the performance (spent less time) to locate the hidden platform compared to OA group. From days 2 to 4, OA group showed reached a plateau in the learning performance (Fig. 3A). In the retention sessions (day 5), OA group showed impaired performance compared to others groups (Fig. 3B). Moreover, there are no statistical differences in the

total distance traveled (Fig. 3C), mean speed (Fig.3D) and in the time spent in the opposite quadrant (Fig.3E) between groups.

3.4 Effect of OA and MN administration in the expression of hippocampal Cdk5, Tau and NR2B proteins

OA infusion increased the hippocampal immunocontent of Cdk5, however three days of pretreatment with MN prevented this increment (Fig.4A). There are no statistical differences in the immunocontent of p39 (Fig.4C) and Calpain-2 proteins (Fig.4D) between groups. Furthermore, there was an increase hippocampal phosphorylation of serine 199/202 of Tau in OA groups, which was also prevented by the pretreatment with MN (Fig.5). However, there was no statistical significant difference between groups regarding the immunocontent of tyrosine 1070 of NR2B subunit (Fig.6B).

3.5 Impact of OA and MN administration in glutamate CSF levels

OA administration significantly enhances CSF glutamate levels. Pretreatment with MN prevent the increase in CSF glutamate levels caused by OA (Fig.6A).

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4. Discussion

The present study aimed to evaluate the potential of a pretreatment with MN as a strategy for preventing AD like alterations induced by intrahippocampal OA infusion in rats. Our results showed consistent signs of neurodegeneration at the local of OA infusion (hippocampus CA1 subfield). The correct location of the infusion was confirmed by immunohistological analysis with DAPI. The

morphological changes observed here were consistent with a previous finding published by Arias et al (2002). No significant differences were observed in the spontaneous locomotion and exploratory activity between groups in the open field task. Indeed, inhibition of PP2A activity by OA seems do not affect locomotory speed, motor coordination and sensorimotor ability (He et al., 2005; Zhang and Simpkins, 2010). Alterations in spontaneous locomotion may have a negative impact on the performance in tasks that requires physical ability. Thus the results of open field allowed engaging animals in the Morris water maze task. Furthermore, hippocampal OA infusion impaired the spatial memory performance on both acquisition and retention phases of water maze task, which was prevented by pretreatment with MN. Increasing number of evidences has suggested that PP2A activity exert a role in learning and memory processes by regulating neuronal homeostasis (Sun et al., 2003). To reiforce this assumption, when an unilateral infusion of OA was performed into rat hippocampus there was a transient impairment of spatial memory on an eight-arm radial maze task (He et al., 2001; He et al., 2005). In addition, Zhang and Simpkins, 2010 reported impaired performance in the Morris water maze task after OA administration.

One of remarkable characteristic of AD is the progressive neurodegeneration associated with cognitive decline (Katzman, 1986; Delbeuck et al., 2003; Ho et al., 2010). The mechanisms underlying brain degeneration and memory deficits in AD comprise the hyperactivation of glutamatergic system due to high levels of glutamate in the synaptic cleft (Hu et al., 2011). OA is a neurotoxin that causes inhibition of PP2A activity, which disrupts the balance between phosphorylation/dephosphorylation favor to a persistent

increasing in intracellular phosphorylation status of regulatory proteins (Bennecib et al., 2000; Sun et al., 2003). This imbalance in the phosphorylation status negatively impact neural cell function and activates neurodegenerative processes (Sun et al., 2003). One possible target of OA neurotoxicity is the persistent phosphorylation of NMDA glutamate receptor subunits and increased glutamate release. We can speculate that under these circumstances there is an increased calcium influx through the ion channel of NMDA receptor. Indeed, we showed that OA increased CSF glutamate levels, suggesting a mechanism that mimics glutamate excitotoxicity. Further, this amino acid is significantly elevated in the CSF of patients with AD (Kaiser et al., 2010). Moreover, it has been described that OA causes an abnormal Tau hyperphosphorylation and neuronal death (Arendt et al., 1998). Here we were able to demonstrate that OA caused an aberrant phosphorylation in serine 199/202 of Tau and the pretreatment with MN significantly prevented this alteration. Interesting, Tau can be downstream phosphorylated by Cdk5 at Serine 199, Serine 202 and Serine 396 (Gong et al., 2005; Liu et al., 2006). In our experimental protocol we found that the hippocampal immunocontent of Cdk5 are increased in OA relative to control group. This increase in the Cdk5 immunocontent was accompanied by an aberrant phosphorylation of serine 199/202 of Tau protein. In contrast, MN pretreatment prevented both, the augment of Cdk5 and aberrant phosphorylation of serine 199/202 of Tau in hippocampus. To date, Cdk5 may have dual physiological roles. For instance, Cdk5 is required for proper development of the mammalian central nervous system (Patrick et al., 1999), as well as can be involved in neuronal death in neurodegenerative diseases (Lew et al., 1994; Tsai et al., 1994). A high influx of calcium into neurons is

determinant for the pathological activity of Cdk5. Moreover, several studies have showed that Cdk5 could be involved in the regulation of phosphorylation state of Nr2a and NR2B subunits of NMDAr and, in consequence, the activity of the receptor (Wang et al., 2003; Zhang et al., 2008; Hu et al., 2011). Therefore, regulation of Cdk5 expression emerges as a novel preventive therapeutic target in AD once it regulates an important components of disease pathogenesis; glutamate levels and tau phosphorylation.

Although the pharmacological efficacy of memantine for AD therapy is well known, the potential neuroprotective effects of pretreatment has been little explored. Recently, Babu and Ramanhathan et al. (2009) showed that a pre-ischemic treatment with MN prevented the excessive glutamate levels in hippocampus caused by the middle cerebral artery occlusion, further preventing neurological deficits. Thus, preventive approaches with MN can also avoid acute neural damage. In this context, there was an increase interest for search new tools, methods and molecular markers to achieve an early diagnosis of AD or to detect, before the symptoms, individual at risk for development of AD (Tan et al., 2007; Reitz and Mayeux, 2009; van Exel et al., 2009; Shoji, 2011; van Harten et al., 2011; Vialatte et al., 2011). Overall, an early diagnosis coupled with a pretreatment could provide opportunities to prevent/delay brain neurochemical and morphological alterations.

In summary, the pretreatment with MN could prevent the spatial memory deficits caused by OA intrahipocampal administration in rats. The mechanisms underlying this neuroprotective effects involves the prevention of the increase in brain glutamate levels along with decrease expression of Cdk5 and, in consequence, downstream phosphorylation of Tau (ser199/202) protein. To

conclude, MN has potential therapeutic role in preventing behavioral and neurochemical alterations caused by an AD like model induced by OA.

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Legends to figure

Figure 1. Damage signs in a representative DAPI imunnohistochemistry of hippocampal slices. (A) Hippocampus slices stained with DAPI (0.0001%; Milipore) showing signs of nuclei degeneration caused by OA in CA1 region.

Figure 2. Open Field Task. No differences in spontaneous locomotion and exploratory activity. (A) Distance traveled minute by minute showing exploratory and locomotor activity. (B) Total distance travelled. (C) Mean speed. (D) Time in central zone. (E) Total distance travelled in central zone. Groups: control (CO), memantine (MN), okadaic acid (OA) and okadaic acid/memantine, n=10 per group. Data were represent in mean±SEM. *p<0.05 between groups.

Figure 3. Morris water maze task. Okadaic acid impairs the spatial memory performance, but memantine prevent this deficit. (A) Acquisition task: the latency to found platform to assess the learning ability. (B) Retention task: the time in the target quadrant to assess the memory retention. (C) Total distance travelled. (D) Mean speed. (E) Time in opposite quadrant. Groups: control (CO), memantine (MN), okadaic acid (OA) and okadaic acid/memantine, n=10 per group. Data were represent in mean±SEM. *p<0.05 between groups.

Figure 4. MN prevents the augment of Cdk5 expression induced by OA. Western blot in hippocampus homogenate of proteins involved in Cdk5 signaling pathways: (A) Immunocontent of Cdk5, (B) Immunocontent of p39 and (C) Immunocontent of Calpain-2. Groups: control (CO), memantine (MN), okadaic acid (OA) and okadaic acid/memantine, n=6 per group. Data were represent in mean±SEM. *p<0.05 between groups.

Figure 5. MN prevents aberrant phosphorylation in Tau protein induced by OA. (A) Western blot of Immunocontent of serine199/202 of tau in hippocampus homogenate. Groups: control (CO), memantine (MN), okadaic acid (OA) and okadaic acid/memantine, n=6 per group. Data were represent in mean±SEM. *p<0.05 between groups.

Figure.6 MN prevents the increase in CSF glutamate levels but did not change the immunocontent of phosphorylation of tyrosine1070 of NR2B

(A) Cerebrospinal fluid analysis of glutamate levels (B) Immunocontent of $NR2B^{Tyr1070}$. Groups: control (CO), memantine (MN), okadaic acid (OA) and okadaic acid/memantine, n=6 per group. Data were represent in mean±SEM. *p<0.05 between groups.

Figures

Figure 1.

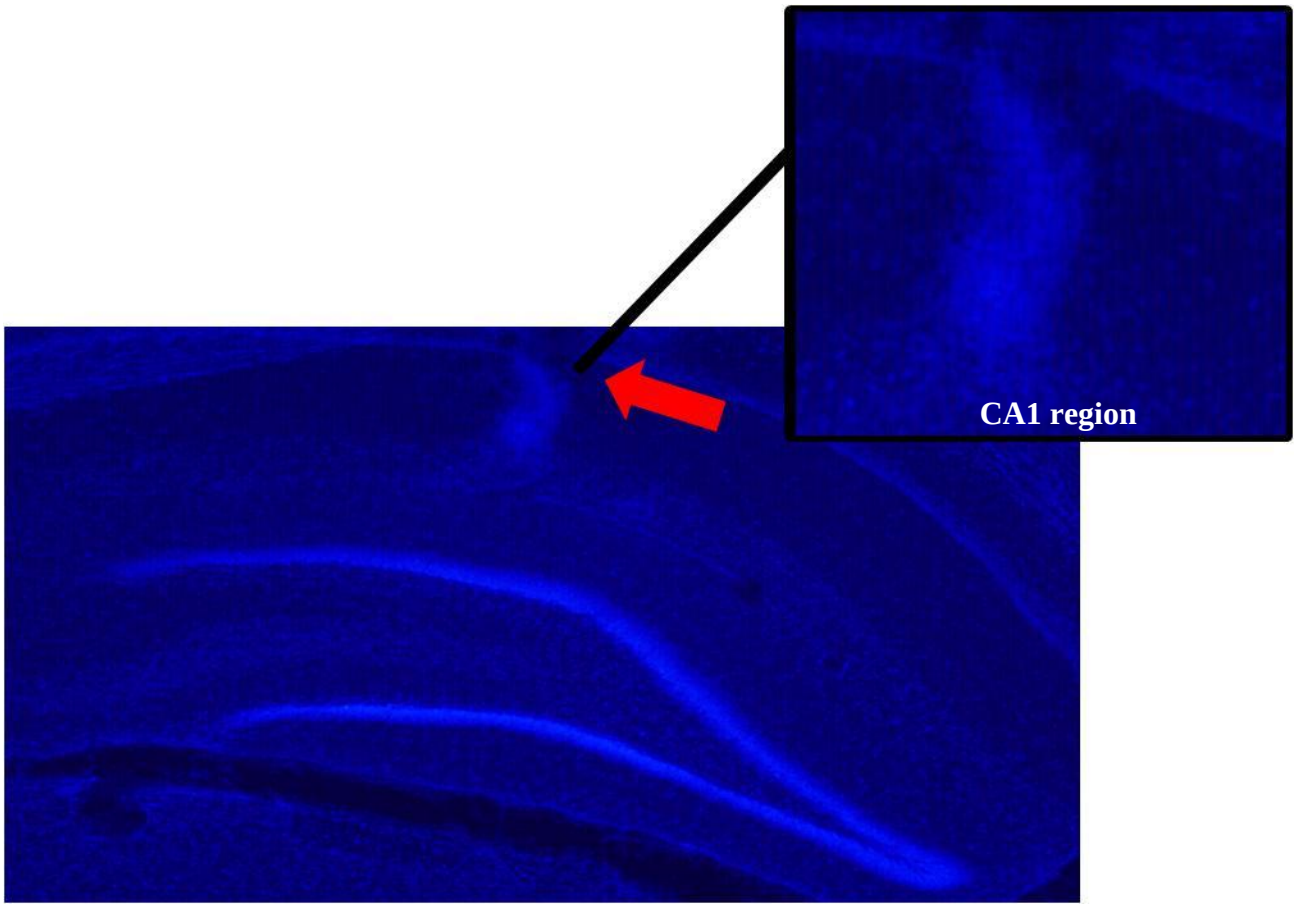


Figure 2.

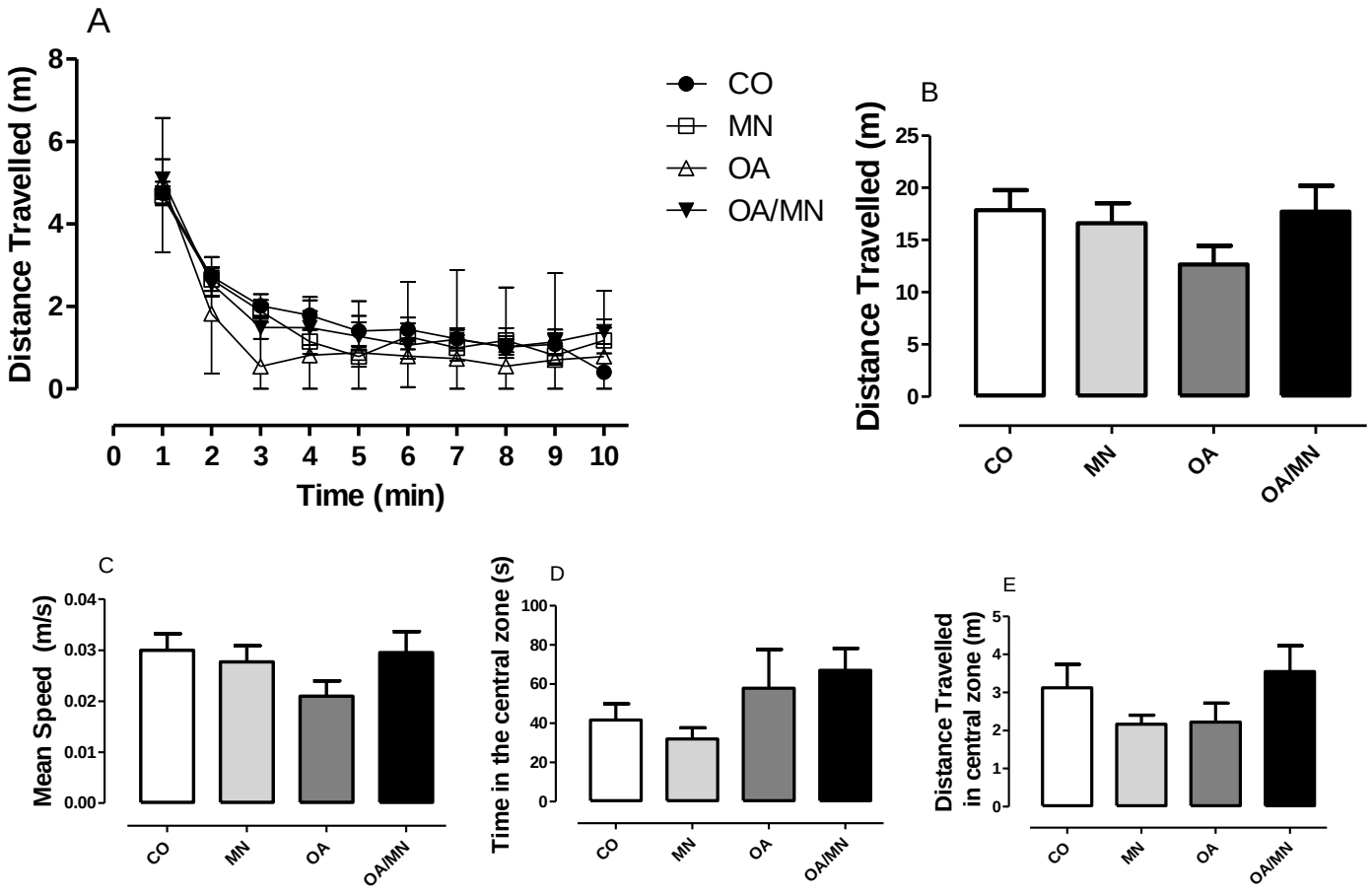
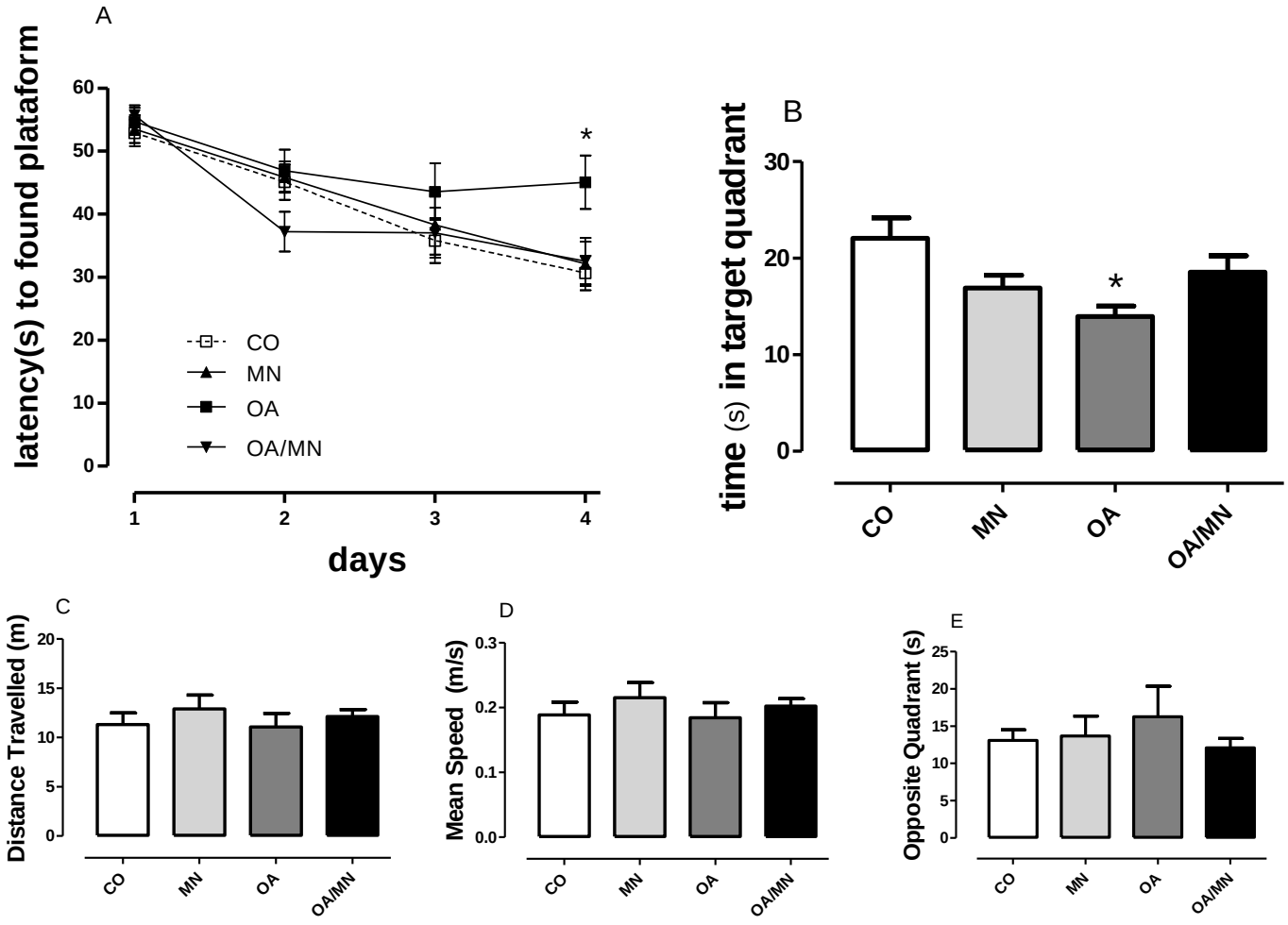

A
Distance Travelled (m)
Time (min)
CO
MN
OA
OA/MN
B
Distance Travelled (m)
C
Mean Speed (m/s)
D
Time in the central zone (s)
E
Distance Travelled in central zone (m)

Figure 3.


A
latency(s) to found plataform
60
50
40
30
20
10
0
1
2
3
4
days
*
CO
MN
OA
OA/MN
B
time (s) in target quadrant
30
20
10
0
*
CO
MN
OA
OA/MN
C
Distance Travelled (m)
20
15
10
5
0
CO
MN
OA
OA/MN
D
Mean Speed (m/s)
0.3
0.2
0.1
0.0
CO
MN
OA
OA/MN
E
Opposite Quadrant (s)
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20
15
10
5
0
CO
MN
OA
OA/MN

Figure 4.

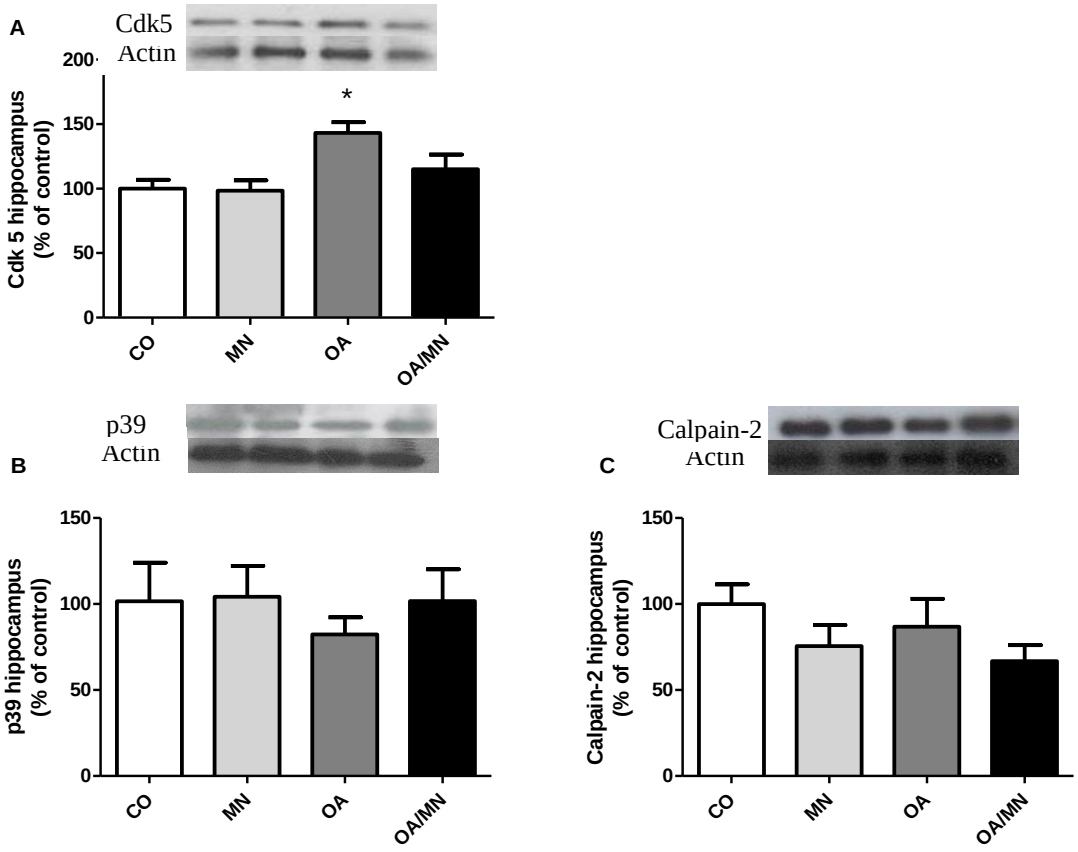

A
Cdk5
Actin
200
150
100
50
0
*
Cdk 5 hippocampus (% of control)
CO
MN
OA
OA/MN
B
p39
Actin
150
100
50
0
p39 hippocampus (% of control)
CO
MN
OA
OA/MN
C
Calpain-2
Actin
150
100
50
0
Calpain-2 hippocampus (% of control)
CO
MN
OA
OA/MN

Figure 5.

A

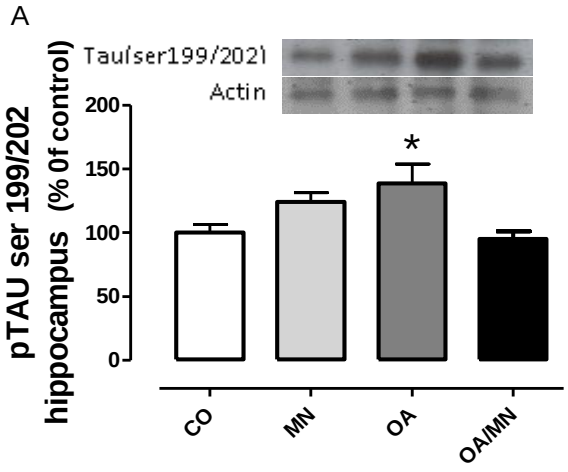
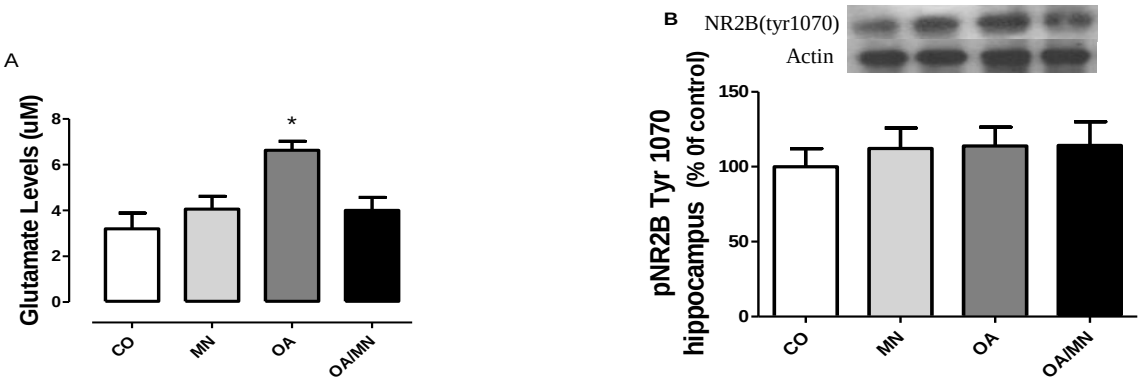

Tau(ser199/202)
Actin
pTAU ser 199/202
hippocampus (% 0f control)
200
150
100
50
0
*
CO
MN
OA
OA/MN

Figure 6.


A
Glutamate Levels (uM)
8
6
4
2
0
*
CO
MN
OA
OA/MN
B
NR2B(tyr1070)
Actin
pNR2B Tyr 1070
hippocampus (% 0f control)
150
100
50
0
CO
MN
OA
OA/MN

Parte III

3. Discussão

A doença de Alzheimer (DA) provoca uma série de alterações no comportamento dos pacientes, incluindo uma diminuição de memória e aprendizado (Katzman, 1986; Ho et al., 2010). Neste trabalho nós avaliamos os efeitos comportamentais de um modelo similar a DA induzido pelo ácido ocadáico (AO), e um possível papel neuroprotetor de um pré-tratamento com memantina (MN).

Nossos resultados não mostraram nenhuma alteração na locomoção e no caráter exploratório nos animais tratados com MN e AO. Estes dados são consistentes com outros trabalhos utilizando o AO como modelo de neurodegeneração, onde não foram observados déficits na coordenação motora (Zhang and Simpkins, 2010). Os déficits no aprendizado e memória dos animais no labirinto aquático de Morris pela infusão de AO em nosso modelo, são muito similares aos descritos em estudos anteriores que mostram que o AO causa um declínio na cognição, memória e aprendizado dos animais (Sun et al., 2003; He et al., 2005; Wu et al., 2008; Zhang and Simpkins, 2010). Estas alterações comportamentais causadas pela inibição da PP2A via AO, foram prevenidas pelo pré-tratamento com MN.

A terapia com MN tem se mostrado eficaz para o controle da progressão dos sintomas da DA, mas também demonstra uma capacidade de melhorar aspectos cognitivos dos pacientes (Winblad and Poritis, 1999; Bakchine and Loft, 2008; Filali et al., 2011). Porém, os efeitos de um pré-tratamento com MN ainda tem sido pouco explorados. Recentemente, Babu and Ramanhathan et

al. (2009) demonstraram que o tratamento com MN antes de um evento isquêmico pode prevenir déficits neurológicos causados pela isquemia em ratos.

Além das alterações comportamentais existem muitos marcadores neuroquímicos clássicos da DA, como a fosforilação aberrante da proteína Tau. A hiperfosforilação da Tau causa o aparecimento de emaranhados neurofibrilares levando a uma desestabilização da rede de microtúbulos e a subseqüentemente a morte neuronal (Grundke-Iqbal et al., 2006; Mazanetz and Fischer, 2007). Nossos resultados mostraram um aumento no imunoconteúdo da proteína Tau fosforilada nos sítios serina 199/202 no grupo AO, que foi prevenido com a MN. A fosforilação desta subunidade é realizada pela ação da Cdk5 e, em nosso modelo de neurodegeneração, houve um aumento do imunoconteúdo da Cdk5, que também foi prevenido com o pré-tratamento com MN. Estes dados apontam para uma possível ligação entre a diminuição da fosforilação da serina 199/202 da proteína Tau e da Cdk5 com o efeito neuroprotetor da MN. Neste caso, a MN poderia estar regulando a fosforilação da tau via diminuição do imunoconteúdo da Cdk5. Estudos recentes relatam que a Cdk5 poderia estar envolvida nos mecanismos patológicos de doenças neurodegenerativas (Lew et al., 1994; Tsai et al., 1994; Alonso et al., 1996; Wen et al., 2004; Ballatore et al., 2007). Além disso, trabalhos demonstram que a Cdk5 pode estar envolvida na modulação da atividade do sistema glutamatérgico especialmente na fosforilação de algumas subunidades do receptor NMDA (Wang et al., 2003; Zhang et al., 2008; Hu et al., 2011), como a fosforilação do sítio tirosina 1472 da subunidade NR2B do receptor NMDA (Zhang et al., 2008; Peng et al., 2009), porém em nossos resultados não houve

alteração no imunoconteúdo na fosforilação da tirosina 1070 do NR2B. Estes dados sugerem que apesar de o AO causar significantes alterações nos mecanismos de sinalização via sistema glutamatérgico estas alterações não envolvem todas as subunidades do receptor NMDA. O sistema glutamatérgico tem sido alvo frequente de pesquisas direcionadas a intervenções farmacológicas para a melhora dos processos de memória e aprendizado. A excitotoxicidade glutamatérgica vem sendo extremamente associada a etiopatogenia de doenças neurodegenerativas, e sua função parece estar bem estabelecida nestes processos (Lau and Tymianski, 2010).

A infusão de AO causou um aumento nos níveis de glutamato no liquido cefalorraquidiano (LCR) dos ratos, o que foi totalmente revertido pelo pré-tratamento com MN. Os níveis deste aminoácido estão significantemente aumentados no LCR de pacientes portadores da DA (Kaiser et al., 2010), assim o modelo mimetiza alguns resultados clínicos. Em resumo nossos dados mostram que a infusão do AO, causa um declínio na memória espacial dos ratos. Este prejuízo está associado ao aumento de glutamato no LCR, aumento da expressão de Cdk5 e do seu alvo molecular, a Tau fosforilada na serina 199/202. O pré-tratamento com MN teve um papel neuroprotetor, pois conseguiu prevenir os danos comportamentais e neuroquímicos causados pelo AO.

Um pré-tratamento torna-se interessante pois, a procura por novas ferramentas para um diagnóstico precoce da DA é um alvo cada vez mais frequente de pesquisas em âmbito mundial (Tan et al., 2007; Reitz and Mayeux, 2009; van Exel et al., 2009). Um diagnóstico precoce acoplado com

um pré-tratamento farmacológico poderia ser uma nova estratégia para pacientes com risco de desenvolverem a DA.

4. Conclusões

O pré-tratamento com MN reverteu os danos cognitivos e alterações neuroquímicas causadas pela infusão de AO. Associada uma futura ferramenta de diagnóstico a MN poderia ser uma alternativa para a prevenção do aparecimento dos sintomas e da progressão da DA em pessoas com risco de desenvolverem esta patologia.

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