UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE
DEPARTAMENTO DE BIOQUÍMICA
CURSO DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA

Alterações bioquímicas e comportamentais em ratos submetidos à administração intra-estriatal de hipoxantina

CAREN SERRA BAVARESCO

Orientadora: Profª. Drª. Angela Terezinha de Souza Wyse

Co-orientador: Prof. Dr. Carlos Alexandre Netto

Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas: Bioquímica da Universidade Federal do Rio Grande do Sul, como requisito parcial para a obtenção do título de Doutor em Bioquímica

Porto Alegre, maio de 2008

Dedico este trabalho à minha família pelo
incentivo, apoio e compreensão

"O conhecimento em si revela-se não apenas a fonte do poder da mais alta qualidade, mas também o mais importante ingrediente da força e riqueza. Colocando de outra maneira, o conhecimento passou de um complemento do poder do dinheiro e do poder dos músculos, para ser sua própria essência. Ele é, de fato, o amplificador absoluto."

Alvin Toffler

AGRADECIMENTOS

À minha querida orientadora e amiga Profª. Drª. Angela T.S. Wyse pela oportunidade, confiança, ensinamentos, paciência e pelo exemplo profissional a ser seguido.

Ao Prof. Dr. Carlos Alexandre Netto pelos ensinamentos.

Aos Profs. Clóvis M. D. Wannmacher, Moacir Wajner e Carlos S. Dutra-Filho pelos ensinamentos e amizade.

Aos colegas do laboratório 34 pelo companheirismo, apoio e amizade.

À Fábria, pela presença constante, companheirismo e amizade durante todos estes anos.

À Izabel M. Maciel e família pelo apoio, incentivo e companheirismo.

Aos meus pais, João Baptista Bavaresco e Rosa Maria Serra Bavaresco, pelo exemplo, incentivo, carinho, amor e confiança.

À minha irmã, Andréa, e minha tia, Liane, pelo incentivo, carinho e apoio.

Aos meus grandes companheiros caninos, Piruvato e Siso, por garantirem minha paz nos piores momentos.

Aos meus amigos pelo apoio e incentivo ao longo destes 4 anos.

A todos os funcionários e professores deste departamento pela atenção.

À Universidade Federal do Rio Grande do Sul (UFRGS) pela possibilidade de estudo.

Ao CNPq, CAPES, FAPERGS, PROPESQ e UFRGS.

Á Deus por tudo!

MUITO OBRIGADA!

RESUMO

A síndrome de Lesch Nyhan é um erro inato do metabolismo das purinas, de característica recessiva, ligado ao sexo. Caracteriza-se, bioquimicamente, pela deficiência na atividade da enzima hipoxantina-guanina fosforribosiltransferase (HGPRT), resultando principalmente no acúmulo tecidual de hipoxantina. O quadro clínico manifestado é bastante característico incluindo alterações motoras e cognitivas, retardo mental, espasticidade e automutilação. Considerando que os mecanismos envolvidos nas alterações cerebrais encontradas nessa síndrome ainda são pouco conhecidos, os objetivos do presente estudo foram investigar o efeito da administração intra-estriatal de hipoxantina sobre parâmetros bioquímicos cerebrais (atividades da Na^+, K^+- ATPase e acetilcolinesterase (AChE), parâmetros de estresse oxidativo, hidrólise dos nucleotídeos da adenina) e comportamentais (tarefas do labirinto aquático de Morris, campo aberto e esquiva inibitória) em ratos. Os resultados mostraram que a administração intra-estriatal de hipoxantina reduziu as atividades das enzimas Na^+, K^+- ATPase e AChE em estriado, hipocampo e no córtex cerebral de ratos. A infusão de hipoxantina aumentou a quimioluminescência,, substâncias reativas ao ácido tiobarbitúrico (TBARS) e atividade da enzima glutationa peroxidase (GPx), e reduziu a capacidade antioxidante tecidual (TRAP) e as atividades das enzimas superóxido dismutase (SOD) e catalase (CAT) em estriado de ratos. As hidrólises dos nucleotídeos da adenina também foram inibidas pela administração de hipoxantina. Os efeitos relatados possivelmente ocorreram através da geração de radicais livres, uma vez que a administração de vitaminas E e C previniu tais efeitos, com exceção do TRAP. Considerando as alterações neuroquímicas induzidas pela administração de hipoxantina observadas em nosso modelo experimental, a próxima etapa desse trabalho foi investigar o papel da administração de hipoxantina sobre a memória/ aprendizagem em ratos na tarefa do labirinto aquático de Morris e esquiva inibitória. A atividade motora dos animais também foi avaliada na tarefa de campo aberto. Os resultados mostraram que a hipoxantina provocou um déficit de

memória/ aprendizado em ambas as tarefas realizadas, contudo não alterou o comportamento motor dos animais. Nossos resultados, em conjunto, mostram que a administração intra-estriatal de hipoxantina provoca uma série de alterações bioquímicas e comportamentais as quais podem, pelo menos em parte, contribuir para as disfunções neurológicas características observadas nesta síndrome. Além disso, se nossas evidências se confirmarem em humanos, a utilização de antioxidantes, tais como as vitaminas E e C, poderão ser utilizados como estratégias terapêuticas a fim de evitar as alterações neurológicas nos pacientes portadores da síndrome de Lesch Nyhan.

ABSTRACT

Lesch Nyhan is an inborn X-linked recessive disease of purine metabolism characterized by deficiency of hypoxanthine-guanine phosphoribosyltranspherase (HGPRT) activity, resulting mainly in tissue accumulation of hypoxanthine. Affected patients present motor and cognitive deficits, spasticity, and self-mutilation behavior. Considering that the mechanisms involved in brain dysfunction found in this syndrome are poorly understood, the general objective of this study was to investigated the effect on intrastriatal hypoxanthine administration on some cerebral biochemical parameters (activities of Na^+, K^+- ATPase and acetylcholinesterase (AChE), oxidative stress parameters, adenine nucleotide hydrolysis) and behavioral (water-maze, step-down inhibitory avoidance and open field tasks) in rats. Results showed that intrastriatal hypoxanthine administration inhibited Na^+, K^+- ATPase and AChE in striatum, hippocampus and cerebral cortex of rats. We also verified that hypoxanthine administration increased chemiluminescence, thiobarbituric acid reactive substance (TBARS) and glutathione peroxidase (GPx) activity and reduced total radical-trapping antioxidant parameter (TRAP) and also superoxide dismutase (SOD) and catalase (CAT) activities in striatum of rats. Moreover, adenine nucleotide hydrolysis was also inhibited by hypoxanthine administration. These effects could be probably related to free radical generation since pretreatment with vitamins E and C prevented those effects, excepting for TRAP. Considering the neurochemical alterations provoked by hypoxanthine administration in this experimental model, the next step in this study was to investigate the effect of intrastriatal hypoxanthine administration on memory/ learning of rats in water-maze and step-down inhibitory avoidance tasks. The motor activity of the rats was evaluated by open field task. Results showed that hypoxanthine administration impaired memory/ learning in both tasks, however the motor activity of rats was not altered. Taken together, our results showed that intrastriatal hypoxanthine administration induced various biochemical and behavioral modification that could contribute, at

least in part, to the characteristically neurological dysfunction observed in this syndrome. Moreover, if our evidences also occur in human, supplementation with antioxidants, like vitamins E and C, could be used as therapeutically strategies in order to avoid the neurological disturbances present in Lesch Nyhan patients.

SUMÁRIO

LISTA DE ABREVIATURAS

AChE – Acetilcolinesterase

AMP – Adenosina 5´- monofosfato

ADP – Adenosina 5´- difosfato

ATP – Adenosina 5´-trifosfato

CAT – Catalse

DNA – Ácido desoxirribonucléico

EIM – Erros inatos do metabolismo

ERO - Espécies reativas de oxigênio

ERN- Espécies reativas de nitrogênio

GMP – Guanina monofosfato

GPx – Glutationa Peroxidase

GR - Glutationa redutase

GSH – Glutationa reduzida

H_2O_2 – Peróxido de Hidrogênio

HGPRT - hipoxantina-guanina fosforribosiltransferase

Hpx – Hipoxantina

IMP – Inosina monofosfato

LPO – Lipoperoxidação

NADPH – Nicotinamida adenina dinucleotídeo fosfato (forma reduzida)

PPRP – Pirofosfato Ribose

$O_2^{\bullet-}$ - Ânion superóxido

$^1O_2$ - Oxigênio “singlet”

OH $^\bullet$ - Radical hidroxila

SNC – Sistema nervoso central

SOD – Superóxido dismutase

TBARS: Substâncias reativas ao ácido tiobarbitúrico

TRAP – Capacidade antioxidante total não-enzimática

INTRODUÇÃO

1.0. INTRODUÇÃO

1.1. Erros Inatos do Metabolismo:

Em 1908, Sir Archibald Garrot introduziu o termo Erros Inatos do Metabolismo (EIM) para as alterações genéticas que se manifestam pela síntese de uma proteína anômala, geralmente uma enzima, ou através da diminuição ou ausência de sua síntese, resultando no bloqueio da rota metabólica envolvida. Como conseqüência, pode ocorrer tanto o acúmulo de metabólitos tóxicos como a falta de produtos essenciais, ambos com doença subseqüente (BICKEL, 1987). Os EIM apresentam-se relativamente freqüentes em seu conjunto, podendo atingir 1 em cada 100 recém-nascidos vivos (GIUGLIANI, 1988). Até o momento, foram descritos mais de 500 EIM (SCRIVER et al., 2001), envolvendo processos de transporte, armazenamento e síntese de biomoléculas.

Os EIM envolvem as mais diferentes áreas do metabolismo. Dentre os erros inatos do metabolismo das purinas, destaca-se a síndrome de Lesch-Nyhan, caracterizada pela deficiência na enzima hipoxantina-guanina fosforribosiltransferase, resultando em acúmulo tecidual de hipoxantina, xantina e ácido úrico (JINNAH & FRIEDMANN, 2001).

1.2. ERROS INATOS DO METABOLISMO DAS PURINAS

1.2.1. Metabolismo das Purinas

As principais bases púricas encontradas nas células são adenina e guanina, as quais podem formar nucleosídeos através da incorporação de ribose ou 2-desoxirribose ao anel purínico. A adição de ésteres de fosfato nesses nucleosídeos resulta na formação de nucleotídeos purínicos que desempenham importantes papéis no metabolismo celular (CORY, 1998), dentre as quais podemos destacar o papel no metabolismo energético

celular, tendo em vista que o ATP é a principal fonte energética, como constituintes monoméricos dos ácidos nucléicos, bem como mediadores fisiológicos.

Paralelamente, outra classe de derivados purínicos, as oxipurinas, tais como hipoxantina e xantina, apresentam um importante papel no metabolismo celular (BALIS, 1976). O fornecimento de purinas para o *pool* celular depende das vias de síntese de novo, recuperação ou através da degradação de nucleosídeos exógenos. A via de síntese de novo das purinas consiste em diversos passos metabólicos, utilizando grande quantidade de ATP celular para fornecimento de energia (ROSSITER & CASKEY, 1995).

A biossíntese de novo das purinas é composta por diversas reações enzimáticas as quais requerem a hidrolise de ATP, culminando com a formação de inosinato (IMP) (LEHNINGER et al., 1995). Por outro lado, a via de recuperação utiliza hipoxantina, guanina e adenina como substrato para a ação de fosforribosiltransferases. Essas enzimas transferem a ribose fosfato do fosforribosilpirofosfato para a base purínica em presença de magnésio. Adenina fosforribosiltransferase (APRTase) converte adenina em AMP, enquanto a enzima hipoxantina – guanina fosforribosiltransferase (HGPRT) age sobre a hipoxantina ou guanina para formar IMP ou GMP, respectivamente. Essa rota de recuperação conserva energia e permite que as células formem nucleotídeos a partir de bases pré-existentes, tendo em vista que 90% das purinas livres geradas durante o metabolismo intracelular provêm da rota de salvação (JINNAH & FRIEDMANN, 2001).

Os níveis intracelulares de purinas também podem ser mantidos através da incorporação de purinas livres e nucleosídeos produzidos no meio extracelular. A incorporação desses compostos depende da atividade das enzimas envolvidas na rota de regeneração das purinas. A via de degradação das purinas envolvem as enzimas 5

´-nucleotidase, purina nucleosídeo fosforilase, xantina oxidase, guanase e adenosina desaminase, obtendo-se como produto final ácido úrico, o qual pode ser facilmente excretado (VISSER at al., 2000, JINNAH & FRIEDMANN, 2001).

A hipoxantina é uma das maiores fontes de purinas passíveis de reutilização nos tecidos de mamíferos (ROSSITER & CASKEY, 1995). A hipoxantina atua como um intermediário ativo nas células, podendo ser degradada pela enzima xantina oxidase, formando xantina ou inosina monofosfato (IMP) pela ação da enzima hipoxantina-guanina fosforribosiltransferase. Esta reação é responsável pela manutenção do "pool" de hipoxantina intracelular, o qual será utilizado na conversão de produtos úteis para as células (BALIS, 1976; VISSER at al., 2000).

1.2.2. Hipoxantina-Guanina Fosforribosiltransferase (E.C. 2.4.2.8)

A enzima hipoxantina-guanina fosforribosiltransferase (HGPRT) apresenta peso molecular de 24.470 Da contendo aproximadamente 218 aminoácidos por monômero, e é responsável pela transferência da fosforribose presente na molécula de PP-ribose-P para a posição nove da hipoxantina ou guanina (ROSSITER & CASKEY, 1995). A HGPRT é composta por duas subunidades (α / ß). A cinética de reação é um mecanismo complexo e dependente do íon Mg^{2+}. PP-ribose-P liga-se inicialmente à enzima, seguido do composto purínico - último composto a ser liberado. Os substratos enzimáticos principais são hipoxantina, guanina e PRPP, entretanto alguns trabalhos sugerem que a HGPRT tenha pouca afinidade pela xantina (SEEGMILLER, 1976).

A HGPRT apresenta-se amplamente distribuída nos tecidos, especialmente no sistema nervoso central (SNC), sendo que, particularmente, os gânglios da base são dependentes da atividade dessa enzima para manutenção dos níveis de purinas teciduais através da via de reutilização (MORIWAKI et al., 1999, VISSER et al., 2000). Mutações

gênicas levam a perda parcial ou total da atividade da HGPRT, reduzindo a afinidade da enzima pela hipoxantina, guanina e PP-ribose-P, com conseqüente acúmulo desses compostos (SEEGMILLER, 1976, ROSSITER & CASKEY, 1995).

Tendo em vista a vital importância dos compostos purínicos e da atividade da enzima HGPRT para o metabolismo das purinas, defeitos enzimáticos ou na via de regeneração das purinas resultam em síndromes. Dentre elas, ressalta-se a síndrome de **Lesch Nyhan**, a qual é caracterizada pela deficiência na enzima HGPRT, pelo acúmulo tecidual de oxipurinas, especialmente a hipoxantina, e por pronunciadas alterações no SNC (JINNAH & FRIEDMANN, 2001).

1.3. SÍNDROME DE LESCH –NYHAN

1.3.1 Conceito e Histórico

A síndrome de Lesch Nyhan é um EIM associado à herança autossômica recessiva ligada ao sexo. Caracteriza-se pela deficiência na atividade da HGPRT a qual catalisa a conversão de hipoxantina e guanina em seus respectivos nucleotídeos IMP e GMP, conseqüentemente a hipoxantina não pode ser convertida a IMP (NYHAN, 1978, MATEOS et al., 1991, VISSER at al., 2000, JINNAH & FRIEDMANN, 2001). Além disso, ocorre um aumento da via de síntese de novo das purinas decorrente da ineficiência na rota de regeneração (TORRES-JIMÉNEZ et al., 1998). Essas alterações acarretam o acúmulo tecidual de xantina, ácido úrico e, principalmente, de hipoxantina.

O primeiro caso da doença foi publicado por Lesch e Nyhan (1964), entretanto a relação entre a deficiência na atividade da HGPRT e a síndrome de Lesch Nyhan somente foi demonstrada em 1967 (SEEGMILLER et al., 1967). Atualmente, essa síndrome apresenta incidência aproximada de 1/ 100.000 nascidos vivos (DeANTONIO et al., 2002).

1.3.2 Sinais e Sintomas

Os sintomas clínicos da doença parecem estar relacionados com a atividade residual da enzima HGPRT. Pacientes portadores da síndrome de Lesch Nyhan geralmente apresentam atividade da HGPRT inferior a 1,5 %. Diversos estudos apontam para as marcantes características genéticas (JINNAH et al., 1990; JINNAH et al., 2004), metabólicas (PUIG et al., 1989; JINNAH & FRIEDMAN, 2001), cognitivas (MATTHEWS et al., 1995; SCHRETLEN et al., 2001) e comportamentais (NYHAN, 1973; CAUWELS & MARTENS, 2005; SCHRETLEN et al., 2005) associadas a esta síndrome. Pacientes afetados por essa doença apresentam retardo mental, alterações motoras e cognitivas, aliado a hiperuricemia, gota e auto-mutilação, a qual é caracterizada por mordeduras de lábios, língua e dedos, com aparente perda tecidual (HENDERSON, 1968, EDWARDS et al., 1979, JINNAH et al., 1990; CAUWELS & MARTENS, 2005; SCHRETLEN et al., 2005, JINNAH et al., 2006).

A manifestação clínica mais característica é a auto-mutilação, a qual ocorre em 85% dos casos da doença (NYHAN, 1973; CAUWELS & MARTENS, 2005). O desenvolvimento desse comportamento inicia, aparentemente, no segundo ano de vida, e coincide com o término de erupção da dentição decídua, tendendo a diminuir com o aumento do autocontrole. Todavia os pacientes apresentam sensibilidade dolorosa e parecem sofrer com essa conduta (VISSER at al., 2000, DeANTONIO et al., 2002).

No SNC, estudos neuroquímicos demonstraram uma redução seletiva do conteúdo dopaminérgico no estriado, similares àqueles encontrados em pacientes portadores do mal de Parkinson (VISSER et al., 2002). Todavia, a correlação entre a deficiência na atividade da enzima HGPRT e a redução dos níveis de dopamina não está esclarecida (VISSER et al., 2000; SHIRLEY et al., 2007). Autores sugerem que o estresse oxidativo

poderia estar envolvido no processo de deteriorização estriatal, através de um mecanismo similar ao encontrado na doença de Parkinson. Além disso, a dependência estriatal da atividade da HGPRT para a manutenção dos níveis de purinas, aliado a diminuição dos prolongamentos dos neurônios dopaminérgicos poderiam contribuir para o aparecimento dessa disfunção (PAULMOR et al., 1989, LOEFFLER et al., 2000, JINNAH & FRIEDMANN, 2001).

1.3.3. Diagnóstico

A análise dos níveis séricos de hipoxantina também contribue para a determinação do diagnóstico definitivo (SWEETMAN, 1968, HARKNESS, 1988, PUIG et al., 1989, MATEOS et al., 1991). Os valores obtidos para esta oxipurina em plasma de indivíduos normais correspondem a 1,7 μM para hipoxantina, entretanto, em pacientes portadores da síndrome de Lesch Nyhan estas concentrações podem atingir valores de 10 μM. (PUIG et al., 1989). As concentrações de hipoxantina também se encontram elevadas no líquido cérebro-espinhal dos indivíduos portadores da doença (SWEETMAN, 1968; VISSER at al., 2000, JINNAH & FRIEDMANN, 2001).

1.3.4 Patogênese

Diversas hipóteses foram formuladas para explicar os mecanismos envolvidos na patogênese da síndrome de Lesch Nyhan, tais como estresse oxidativo, deficiência na síntese de metabólitos essências, aumento na síntese de novo das purinas, bem como o acúmulo de metabólitos tóxicos (JINNAH & FRIEDMANN, 2001). Evidências na literatura indicam uma relação entre a patogênese da síndrome de Lesch Nyhan e os metabólitos acumulados na deficiência da HPGRT (GEDYE, 1992, MA et al., 2001). O aumento na produção e excreção de ácido úrico, assim como o acúmulo das oxipurinas

hipoxantina e xantina, poderia causar um efeito tóxico para o SNC (KISCH et al., 1985, GEDYE, 1992, MA et al., 2001, LOEFFLER et al., 2000, JINNAH & FRIEDMANN, 2001).

Neste contexto, Palmour e colaboradores (1989) demonstraram que o acúmulo de hipoxantina, o principal metabólito acumulado nos tecidos de pacientes com Lesch Nyhan provoca depleção de dopamina em cultura de células neuronais (PALMOUR et al.,1989). Além disso, hipoxantina pode ligar-se a receptores benzodiazepinicos e interferir na função normal desses receptores (TICKU & BURCH, 1980). Neste contexto, estudos indicam que hipoxantina é capaz de provocar reduções no transporte de adenosina em linfócitos (PRIOR et al., 2007). Além disso, evidências mostram alterações nos receptores para adenosina em cérebro de ratos nocaute para HPGRT (BERTELLI et al., 2006). Estudos realizados em nosso grupo demonstram que a hipoxantina *in vitro* inibe significativamente a atividade da Na^+, K^+, ATPase e induz estresse oxidativo em estriado de ratos (BAVARESCO et al., 2004; BAVARESCO et al., 2005). Entretanto, os precisos mecanismos pelos quais a hipoxantina atuaria no SNC e o exato papel desta oxipurina no dano neurológico presente na Lesch Nyhan ainda permanecem desconhecidos.

1.3.5. Tratamento

Em geral, as terapias usadas para a síndrome de Lesch Nyhan apresentam um enfoque estritamente sintomático. O uso de alopurinol (VISSER et al., 2000, DeANTONIO et al., 2002), 5-hidroxitriptofano (DeANTONIO et al., 2002), antagonistas de receptores dopaminérgicos D_2 (DeANTONIO et al., 2002), bem como exercícios de relaxamento e autocontrole frente às situações estressantes (BULL & LaVECCHIO, 1978; OLSON & HOULIHAN, 2000; DeANTONIO et al., 2002),

pulpotomia seguida da remoção das coroas dentárias (LEE et al., 2002), e a utilização de protetores bucais resinosos, a fim de evitar a perda tecidual provocados pela automutilação também estão descritas (CUSUMANO et al., 2001, ROBEY et al., 2003, FARDI et al., 2003; CAUWELS & MARTENS, 2005).

1.4. Na^+, K^+ - ATPase (ATP fosfoidrolase, EC 3.6.1.3).

A Na^+, K^+, ATPase é uma proteína integral de membrana, a qual é essencial para o funcionamento normal de todas as células dos mamíferos. Esta enzima é responsável pelo transporte ativo dos íons Na^+ e K^+ através da membrana plasmática, gerando um gradiente eletroquímico através da membrana (LINGREL & KUNTZWEILER, 1994). Ela utiliza a energia proveniente da hidrólise da ligação do fosfato terminal do ATP (KAPLAN, 2002).

Um grande número de funções celulares está acoplado à manutenção das concentrações intracelulares e extracelulares de Na^+ e K^+, tais como controle do volume celular, excitabilidade neuronal, atividade de enzimas citosólicas, contração musculare, além de auxiliar no movimento de outros íons e compostos através da membrana, como, por exemplo, neurotransmissores (VASILETS & SHWARZ, 1993, BERTORELLO & KARTZ, 1995; KAPLAN, 2002). A regulação da atividade da Na^+, K^+ - ATPase é um processo complexo que requer diferentes níveis de regulação, classificados em mecanismos de “curta duração”, que envolvem a ação de efetores na enzima presente na membrana plasmática, e mecanismos de “longa duração”, os quais provocam alterações na taxas de síntese e/ou degradação enzimática (BERTORELLO & KATZ, 1995, CORNELIUS & MAHMMOUD, 2003).

Quanto a estrutura, a Na^+, K^+ - ATPase é formada por duas subunidades polipeptídicas principais (α e ß), associadas covalentemente e incorporadas no interior

da bicamada lipídica (SKOU & ESMANN, 1992). Recentes trabalhos têm demonstrado a presença de uma terceira subunidade (γ), que está presente, predominantemente, no tecido renal (THERIEN et al., 1997, PU et al., 2002).

A subunidade α está associada às propriedades catalíticas e transportadoras enzimáticas, bem como o sítio de ligação para glicosídios cardiotônicos e cátions (VASILETS & SCHWARZ, 1993; CORNELIUS & MAHMMOUD, 2003). Até o presente momento, foram descobertas 4 isoformas da subunidade α, denominadas α 1, α 2, α 3 e α 4, distribuídas em diferentes proporções nos tecidos. A isoforma α 1 está amplamente distribuída nas células, α 2 é predominante na glia, α 3 nos neurônios (HABIBA et al., 2000) e α 4 presente seletivamente nos testículos (BLANCO & MERCER, 1998).

A subunidade ß possui três isoformas ß (ß1, ß2, ß3) as quais apresentam estruturas básicas compostas de uma região citoplasmática NH_2-terminal, segmento transmembrana, um largo domínio extracelular e 3 pontes dissulfito (SWEADNER, 1991; BLANCO & MERCER, 1998). Essa subunidade parece atuar como chaperonina específica, favorecendo a correta inserção do complexo αß na membrana plasmática, além de aumentar a resistência contra proteólise e degradação celular das subunidades α recém sintetizadas (HASLER et al, 2001). A sensibilidade da Na^+, K^+ - ATPase aos íons Na^+ e K^+ é dependente da isoforma da subunidade ß, devido a sua interferência na ativação enzimática desencadeada pela presença extracelular do íon K^+ (LOPINA, 2001). Evidências sugerem que a redução nas pontes dissulfito, presentes na subunidade ß ou no sítio ativo da subunidade α, provoca uma diminuição na atividade da Na^+, K^+ - ATPase (SWEADNER, 1991; CORNELIUS & MAHMMOUD, 2003). Além disso, no SNC, a subunidade ß parece estar relacionada com a adesão celular (GLOOR et al., 1990; KITAMURA et al., 2005).

Os alicerces para o entendimento dos mecanismos de ação da Na^+, K^+ - ATPase foram embasados em estudos sobre os mecanismos de transporte de cátions, propriedades bioquímicas dos metabólitos intermediários da fosfoenzimas e mudanças conformacionais em proteína dependentes da fosforilação enzimática (KAPLAN, 2002, JORGENSEN et al., 2003). O mecanismo de reação é dependente de dois estados conformacionais associados à fosforilação. Inicialmente, K^+ é liberado na porção intracelular, seguido da subseqüente ligação de 3 íons Na^+ em um sítio de alta afinidade para o Na^+ na porção citoplasmática da enzima favorece a ligação do ATP em seu sítio de ligação e conseqüente fosforilação. A fosforilação facilita a oclusão dos 3 íons Na^+ na molécula da enzima e seu transporte para o meio extracelular. (GLYNN, 1993). Após a liberação dos íons Na^+, há mudança do estado conformacional da Na^+, K^+ - ATPase. O íon K^+ se liga em um sítio na porção extracelular e esta ligação transfere o grupo fosfato liberado para a água (formando o fosfato inorgânico). A enzima é desfosforilada, liberando o íon K^+ na porção intracelular, dando reinício ao ciclo (JORGENSEN et al., 2003).

A inibição da atividade da Na^+, K^+ - ATPase está relacionada a diversas patologias do SNC tais como isquemia cerebral (WYSE et al., 2000; VILLA et al., 2002; CHEN et al., 2007), epilepsia (GRISAR, 1984), doença de Parkinson (KUMAR & KURUP, 2002) e em outras doenças neurodegenerativas (LEES et al., 1993). Evidências também demonstram que a prolongada inibição dessa enzima, causada pela administração de ouabaína, favorece a morte celular (LEES et al., 1993; XIAO et al., 2002; CAPELLA et al., 2001). Adicionalmente, a inibição da Na^+, K^+ - ATPase induz a liberação de neurotransmissores, dentre eles o glutamato, o qual está envolvido na morte neuronal (WESTERINK et al, 1990; CAMACHO & MASSIEU, 2006, NANITSOS et al., 2005).

Além disso, estudos realizados em nosso grupo demonstram que concentrações de hipoxantina *in vitro*, similares àquelas encontradas em pacientes com Lesch Nyhan, inibiram significativamente a atividade da enzima Na^+, K^+, ATPase em membrana plasmática sináptica de estriado de ratos neonatos (BAVARESCO et al., 2004; BAVARESCO et al., 2005). A hipoxantina também é capaz de aumentar os níveis de lipoperoxidação (BECKMAN et al., 1987; BAVARESCO et al., 2005), assim como reduzir as defesas antioxidantes teciduais (BAVARESCO et al., 2005) em cérebro de ratos. Nesta perspectiva, o efeito inibitório de radicais livres sobre a atividade da Na^+, K^+ - ATPase está amplamente descrito na literatura (SIEMS et al., 1996; DOBROTA et al., 1999; BAVARESCO et al., 2005; STEFANELLO et al., 2007; SIMINTZI et al., 2007, ZUGNO et al., 2007).

1.5. Radicais Livres e Estresse Oxidativo

Os radicais livres são, por definição, quaisquer moléculas ou fragmentos de moléculas com um ou mais elétrons desemparelhados, com capacidade para existir de forma independente (HALLIWELL, 2006). A presença de elétrons desemparelhados confere considerável reatividade, viabilizando as ações deletérias mediadas por estas espécies químicas em alvos biológicos tais como lipídios, proteínas e DNA (VALKO et al., 2007). Neste contexto, destacam-se as espécies reativas de oxigênio (ERO) bem como as de nitrogênio (ERN). Dentre as EROs, aponta-se o ânion superóxido ($O_2^{\bullet -}$), peróxido de hidrogênio (H_2O_2), o oxigênio "singlet" ($^1O_2$) e o radical hidroxila ($OH^{\bullet}$). O óxido nítrico e o peroxinitrito são exemplos de ERNs (HALLIWELL, 2006).

A produção do ânion superóxido ocorre principalmente na mitocôndria, pela adição de um elétron ao oxigênio durante o processo de transdução de energia na cadeia de transporte de elétrons (VALKO et al., 2007). Este radical livre pode sofrer

dismutação através da ação da enzima superóxido dismutase (SOD), gerando peróxido de hidrogênio. Por outro lado, depois de formado, o peróxido de hidrogênio é decomposto através da ação das enzimas catalase (CAT) e glutationa-peroxidase (GPx). Em algumas circunstâncias, contudo, o excesso na formação, bem como a diminuição na decomposição do peróxido de hidrogênio pode gerar OH ˙, o qual é extremamente reativo e não possui um sistema eficaz de remoção nos tecidos (MORENO et al., 1995; VALKO et al., 2007).

A formação de radicais livres também está associada a muitos processos fisiológicos que executam funções biológicas importantes. Evidências apontam para a participação destas espécies reativas em processos de sinalização celular (GENESTRA, 2007), síntese e regulação protéica (MAULIK, 2001), nos processos inflamatórios (DRÖGE, 2002) e em eventos mitogênicos (VALKO et al., 2007). Em altas concentrações, os radicais livres apresentam um papel tóxico para as células. Neste contexto, são amplamente discutidas na literatura as alterações nocivas provocadas pelos radicais livres em lipídios, proteínas e também no DNA (HALLIWELL, 2006; VALKO et al., 2007).

Lipoperoxidação (LPO) é o termo utilizado para identificar as reações oxidativas em cadeia geradas pelas EROs e ERNs em lipídios insaturados presentes nas membranas celulares, culminando com as alterações na fluidez, permeabilidade e seletividade das membranas, além de causar danos às proteínas inseridas nesta bicamada lipídica, como enzimas e receptores. Após estas reações de LPO, a formação de malondialdeído é observada e este é um reconhecido marcador para avaliar os processos de LPO (OHKAWA et al., 1979).

As proteínas também podem sofrer danos oxidativos mediados por radicais livres, principalmente nos resíduos de cisteína (-SH), resultando em alterações

conformacionais e funcionais da proteína danificada. Além disso, o DNA também é um importante alvo biológico para a ação deletéria dos radicais livres (HALLIWELL, 2006). Dessa forma, parece ser de suma importância a suplementação de antioxidantes com intuito de conter as alterações induzidas pelos radicais livres nos sistemas biológicos (HALLIWELL, 2006).

Os antioxidantes podem ser definidos, segundo Halliwell (2006), como substâncias que, mesmo em baixas concentrações em relação ao substrato, são capazes de retardar, prevenir ou impedir a oxidação deste substrato. Neste contexto, os antioxidantes atuam como protetores da oxidação de biomoléculas, bem como impedem a propagação da LPO (FLOYD, 1999). De modo geral, os antioxidantes podem ser divididos em antioxidantes enzimáticos e não-enzimáticos (HALLIWELL, 2006).

Dentre as defesas enzimáticas antioxidantes, destacamos a SOD, CAT e GPx. A SOD é uma metaloenzima responsável pela dismutação do ânion superóxido gerando peróxido de hidrogênio e oxigênio (MARKLUND, 1985; HALLIWELL, 2006). Nesta perspectiva, o peróxido de hidrogênio, formado pela ação da SOD, é removido pela ação das enzimas CAT e GPX, resultando em H_2O e O_2 (HALLIWELL, 2006). Além disso, GPx pode reduzir lipoperóxidos de membrana e, desta forma, impedir a propagação das reações em cadeia envolvidas na LPO (MICHIELS et al., 1994; HALLIWELL, 2006).

Por outro lado, dentre as defesas antioxidantes não-enzimáticas destacam-se o α-tocoferol (vitamina E), ácido ascórbico (vitamina C) e a glutationa reduzida (GSH). A GSH é um tripeptídeo formado por glutamato, cisteína e glicina, atuando como substrato da enzima GPX, além de participar na prevenção da oxidação de grupamentos sulfidrila (-SH) presentes em proteínas (HALLIWELL, 2006). Pela oxidação da GSH, obtém-se a glutationa oxidada (GSSG) a qual é eficientemente reduzida até GSH pela enzima

glutationa redutase (GR), a qual requer como co-fator a presença de NADPH (WENDEL, 1981; HITCHLER & DOMANN, 2007).

Aliada à ação da glutationa, o α – tocoferol (vitamina E) atua bloqueando a propagação da cascata de peroxidação ao longo da membrana celular (CHOI, 1993) uma vez que os agentes oxidativos atacam preferencialmente o α-tocoferol em detrimento das membranas celulares (KELLY, 1998). Alguns autores sugerem um papel preventivo e terapêutico do α – tocoferol em doenças neurodegenerativas como nas doenças de Parkinson e de Alzheimer (CHOI, 1993, LOPEZ & BECKER, 2002, BEAL, 2002). Evidências apontam para a participação do ácido ascórbico nos mecanismos envolvidos na redução do α-tocoferol (KELLY, 1998; FREI, 1999; MONTEIRO et al., 2007).

Estresse oxidativo é considerado o processo onde ocorre o distúrbio entre a formação de radicais livres e a captação pelas defesas antioxidantes. O aumento excessivo na produção de radicais livres, a diminuição das defesas antioxidantes, ou ambas as situações, podem induzir o estresse oxidativo que tem sido envolvido nas patogenias das doenças neurodegenerativas, tais como as doenças de Alzheimer, Parkinson e Huntington (HALLIWELL, 1996; HALLIWELL, 2006). Aliados a esses fatores, resultados de nosso grupo evidenciaram o papel do estresse oxidativo em modelos animais de erros inatos do metabolismo (WYSE et al., 2002; MATTÉ et al., 2007; STEFANELLO et al., 2007; DELWING et al., 2007a).

O cérebro, por sua vez, é particularmente suscetível ao estresse oxidativo, uma vez que contém grandes quantidades de lipídios insaturados e apresenta um alto consumo de oxigênio (REZNICK & PACKER, 1993, CHOI, 1993, HALLIWELL, 1996; FLOYD, 1999, HALLIWELL, 2006). Além disso, o cérebro apresenta baixos níveis de defesas antioxidantes enzimáticas e não-enzimáticas (REZNICK & PACKER, 1993, CHOI, 1993, HALLIWELL, 2006; VALKO et al., 2007). Neste sentido, estratégias têm sido

elaboradas para tentar prevenir os danos causados pelo estresse oxidativo, das quais se destaca a utilização de agentes antioxidantes incorporados na dieta dos indivíduos, tais como as vitaminas E e C (HALLIWELL, 2006).

1.6. Acetilcolinesterase

A acetilcolinesterase (AChE) (EC 3.6.1.37), é a enzima envolvida na hidrólise de acetilcolina, localizada preferencialmente em neurônios e axônios. (GRAFIUS et al., 1971). A AChE apresenta-se de duas formas: forma assimétrica (forma A), contendo o sítio catalítico ancorado na membrana, e forma globular (forma G), contendo um ou quatro sítios catalíticos (G1 monomérica, G2 dimérica, G4 tetramérica). No cérebro, a AChE está presente nas formas G1 e G4, em proporções dependentes da região cerebral (LANE et al., 2006). A atividade da AChE têm sido relacionada com ações colinérgicas e não colinérgicas tais como, por exemplo, o crescimento axonal e a sinaptogênese (LASSITER et al., 1998), bem como em processos neurodegenerativos (HENDERSON et al., 1996; GARCIA-ALLOZA et al., 2006). Além disso, modificações nas funções colinérgicas estão associadas a prejuízos na memória/aprendizado (GARCIA-ALLOZA et al., 2006).

Estudos demonstram que a AChE pode ser inibida por radicais livres (DELWING et al., 2005) e que esta inibição pode ser prevenida através da administração de antioxidantes (MONTEIRO et al., 2007).

1.7. ATP-difosfoidrolase (Apirase, NTPDase, EC3.6.1.5) e 5´ Nucleotidase (EC 3.1.3.5)

Muitos trabalhos têm demonstrado que os nucleotídeos como ATP, ADP e adenosina são substâncias sinalizadoras extracelulares no cérebro e em outros tecidos. No tecido

nervoso, pesquisas têm sido dedicadas ao estudo das enzimas do grupo das ectonucleotidases, podendo-se destacar a ecto-ATP difosfoidrolase ou ecto-apirase e a enzima 5`-nucleotidase. Estas enzimas parecem ter um papel essencial na regulação das concentrações de ATP, ADP, AMP e adenosina na fenda sináptica (ZIMMERMANN, 2006). A hidrólise extracelular de nucleotídeos possui fundamental importância não somente na transmissão sináptica, mas também na sinalização celular, sobrevivência e diferenciação neuronal (ZIMMERMANN, 2006).

A ecto-apirase de cérebro humano é uma proteína integral de membrana com dois domínios transmembrana, regiões NH2- e COOH- terminal (ZIMMERMMAN, 2006). As ecto-apirases, de modo geral, apresentam o sítio de hidrólise dos nucleotídeos voltado para o espaço extracelular, subunidade catalítica altamente glicosilada, atividade dependente de cátion divalentes, insensibilidade a inibidores específicos de ATPase e habilidade para hidrolisar nucleotídeos púricos e pirimidínicos (BONAN et al. 1999; ZIMMERMAN, 2006). No SNC, podemos destacar a presença de três NTPDases, as quais diferem entre si na preferência pelo substrato: a NTPDase 2 hidrolisa preferencialmente ATP; a NTPDase 1 hidrolisa ATP e ADP na mesma proporção e a NTPDase 3 degrada ATP em AMP, resultando em um acúmulo transitório de ADP (NEDELIJKOVIC et al., 2006).

A 5´-nucleotidase é proteína tetramérica capaz de realizar a hidrólise do AMP até adenosina, estando sua função catalítica associada á ação da ecto-apirase (BATTASTINI et al., 1995; BONAN et al., 1999). Esta enzima está ancorada na membrana plasmática através de ligação com glicosil-fosfatidilinositol (GPI), estando associada, no SNC, tanto a glia quanto a célula neuronal (ZIMMERMMAN, 2006). Apresenta como inibidores competitivos o ATP e o ADP e sua atividade está relacionada preferencialmente à hidrólise do AMP, resultando na produção de adenosina, molécula com propriedades

neuroprotetoras (ZIMMERMMAN, 2006). A adenosina é um conhecido neuromodulador, o qual executa suas funções através da ligação com os receptores A_1, A_{2A}, A_{2B} e A_3 (RIBEIRO et al., 2003).

Estudos têm demonstrado alterações na atividade das ectonucleotidases em condições patológicas (BONAN et al., 1998; ZIMMERMANN, 2006). Neste contexto, diferentes trabalhos demonstraram o aumento nas atividades das ectonucleotidases em modelos animais de epilepsia (COGNATTO et al., 2007), hipotireoidismo (BRUNO et al., 2005) e diabetes melito (LUNKES et al., 2004). Por outro lado, nosso grupo mostrou a inibição das atividades da ecto-apirase/5´-nucleotidase em hipocampo de ratos submetidos à administração de arginina (DELWING et al., 2007b), prolina (DELWING et al., 2007c) e homocisteína (BÖHMER et al., 2004). Estudos demonstram que as enzimas ecto-apirase e a enzima 5`-nucleotidase podem ser inibidas por radicais livres (VIETTA et al., 1996).

1.8. Memória

A capacidade de formar, manter e utilizar as memórias é uma propriedade fundamental do cérebro e pode ser influênciada por e/ou associada a experiências específicas, tais como emoções, habilidades adquiridas através de treino e de episódios específicos. Quatro etapas fundamentais estão associadas a este processo: aquisição, consolidação, armazenamento das informações e evocação, que é utilizada para a avaliação da memória após processos de aprendizagem (IZQUIERDO & MEDINA, 1997; IZQUIERDO et al., 2006). Em todas estas etapas, modificações celulares e neuroquímicas são induzidas através dos mecanismos de plasticidade sináptica (BRUEL-JUNGERMAN et al., 2007).

As memórias podem ser classificadas de várias maneiras, tais como, a memória espacial, memória aversiva e a memória motivada por medo. Para todas estas situações, ocorre uma integração entre as diferentes estruturas cerebrais a fim de garantir o estabelecimento dos processos relacionados com a memória. De acordo com as teorias atuais, as memórias são processadas como características espaço-temporal dinâmicas através de atividades celulares sincronizadas dentro da trama neuronal, resultando em processos de conectividade entre neurônios co-ativados (BRUEL-JUNGERMAN et al., 2007). Neste contexto, evidências na literatura apontam para o envolvimento do glutamato, GABA, bem como de proteínas cinases nos processos iniciais de formação da memória, seguidos de mecanismos hormonais e neuro-humorais (IZQUIERDO & MEDINA, 1997; WHITE & MCDONALD, 2002; CAMMAROTA et al., 2005). Lesões em estruturas centrais, tais como o estriado, podem alterar as fases de aquisição, consolidação e evocação da memória (MEDINA et al, 1999). Desta forma, o estudo detalhado das alterações induzidas pelos processos supracitados de aquisição, consolidação, armazenamento e evocação da memória são de fundamental importância tendo em vista o crescente número de patologias associadas a déficit cognitivo.

Para estudar os mecanismos envolvidos com a memória, diversas tarefas experimentais têm sido propostas. Dentre elas, a tarefa do labirinto aquático de Morris, é um teste comportamental para avaliação da memória espacial amplamente utilizado na literatura (D´HOOGE & DE DEYN, 2001; DELWING et al., 2006). Esta tarefa consiste em um tanque circular preenchido com água contendo uma plataforma submersa. Nas paredes, pistas são colocadas a fim de servir como ponto de referência para o animal Após treinamento, os animais devem ser capazes de encontrar o local da plataforma, orientando-se espacialmente. Este experimento constitui-se em uma ferramenta

importante para avaliação da memória espacial de referência e de trabalho em ratos (D´HOOGE & DE DEYN, 2001).

A tarefa de campo aberto é realizada em uma caixa de madeira, contendo a parede frontal em vidro, cujo piso está dividido em 12 quadrados. Os animais são colocados na caixa e observados ao longo de 2 minutos. Para a análise desta tarefa, é obtido o número de cruzamentos entre os quadrados, a latência para deixar o primeiro quadrado, bem como o número de bolos fecais e de elevações corporais (os animais mantêm-se sustentados apenas pelas patas traseiras). Esta tarefa é utilizada para a análise da atividade motora do animal (NETTO et al., 1993; KARL et al., 2003).

A tarefa de esquiva inibitória compreende o aprendizado de uma tarefa aversiva, onde os animais são submetidos a um choque de baixa intensidade durante o treinamento. Na sessão de teste, avalia-se a latência do animal para deixar a plataforma e, desta maneira, avalia-se a memória (IZQUIERDO et al., 2002). A tarefa de esquiva inibitória é uma importante ferramenta para analisarmos, distintamente, a ação das drogas nos estágios de formação da memória em diferentes estruturas cerebrais (IZQUIERDO et al., 2002).

1.9. OBJETIVOS GERAIS

Partindo da observação que: a) os pacientes portadores da Lesch Nyhan apresentam disfunções neurológicas cuja gênese ainda é desconhecida; b) evidências na literatura mostram que as oxipurinas, em concentrações semelhantes às encontradas em pacientes com Lesch-Nyhan, possuem efeitos neurotóxicos; c) a hipoxantina é o principal metabólito acumulado na doença de Lesch Nyhan; d) estudos prévios demonstraram que hipoxantina *in vitro* inibe a atividade da Na^+, K^+- ATPase e induz estresse oxidativo em estriado de ratos, o presente trabalho teve como **objetivo geral**

investigar o efeito da administração intra-estriatal de hipoxantina sobre alguns parâmetros bioquímicos cerebrais (atividade da Na^+, K^+- ATPase e acetilcolinesterase, parâmetros de estresse oxidativo, hidrólise de nucleotídeos da adenina) e comportamentais (tarefas do labirinto aquático de Morris, campo aberto e esquiva inibitória) em ratos. Também investigamos o efeito das vitaminas E e C sobre as alterações bioquímicas em ratos submetidos à administração intra-estriatal de hipoxantina. Este trabalho será dividido em **seis capítulos** que correspondem a seis artigos científicos:

Capítulo I

Objetivos específicos

1. Investigar a influência da administração intra-estriatal de hipoxantina sobre a atividade da Na^+, K^+-ATPase em membrana plasmática sináptica de estriado, hipocampo e córtex cerebral de ratos.

2. Verificar o efeito da administração intra-estriatal de hipoxantina sobre parâmetros de estresse oxidativo denominados quimiluminescência, a capaciddae antioxidante tecidual (TRAP) e sobre o conteúdo tiólico total reduzido em homogeneizado de estriado, hipocampo e córtex cerebral de ratos.

Capítulo II

Objetivos específicos

1. Verificar o efeito *in vitro* da hipoxantina sobre as atividades das enzimas Na^+, K^+- ATPase, AChE e CAT em estriado de ratos.

2. Investigar o efeito da administração intra-estriatal de hipoxantina sobre as atividades da Na^+, K^+- ATPase, AChE e CAT em diferentes períodos após a infusão da droga em estriado, hipocampo e córtex cerebral de ratos.

Capítulo III

Objetivos específicos

1. Investigar a influência do tratamento com as vitaminas E e C sobre os efeito mediados pela administração intra-estriatal de hipoxantina sobre as atividades da Na^+, K^+- ATPase, SOD, CAT e GPx em estriado de ratos.
2. Investigar a influência do tratamento com as vitaminas E e C sobre parâmetros de estresse oxidativo denominados substâncias reativas ao ácido tiobarbitúrico (TBARS) e TRAP em estriado de ratos submetidos à administração intra-estriatal de hipoxantina.

Capítulo IV

Objetivos específicos

1. Investigar o efeito da administração intra-estriatal de hipoxantina sobre a hidrólise do ATP, ADP e AMP em sinaptossoma de estriado de ratos em diferentes tempos após a infusão da droga.
2. Verificar o efeito da administração intra-estriatal de hipoxantina sobre as expressões relativas das enzimas NTPDase 1, 2 e 3 e 5´-nucleotidase em estriado de ratos.
3. Verificar a participação do estresse oxidativo, através da técnica do TBARS, nas alterações provocadas pela administração intra-estriatal de hipoxantina na hidrólise do ATP, ADP e AMP em estriado de ratos.

4. Investigar o efeito do pré-tratamento com as vitaminas E e C sobre a hidrólise do ATP, ADP e AMP e sobre o TBARS em ratos submetidos à administração intra-estriatal de hipoxantina.
5. Verificar o efeito *in vitro* da hipoxantina sobre a hidrólise do ATP, ADP e AMP em sinaptossoma de estriado de ratos.

Capítulo V

Objetivos específicos

1. Verificar o efeito da administração intra-estriatal de hipoxantina sobre a memória espacial e na memória de trabalho em ratos na tarefa do labirinto aquático de Morris.
2. Investigar o efeito da administração intra-estriatal de hipoxantina sobre a atividade locomotora de ratos na tarefa do campo aberto.
3. Estudar os níveis de monoaminas no estriado de ratos após a administração intra-estriatal de hipoxantina.

Capítulo VI

Objetivos específicos

1. Verificar o efeito da administração intra-estriatal de hipoxantina sobre a tarefa da esquiva inibitória em ratos.

MATERIAIS, MÉTODOS E RESULTADOS

3.1. Modelo Experimental

O modelo experimental desenvolvido em nosso estudo baseia-se na infusão de hipoxantina diretamente no estriado, utilizando como recurso a cirurgia estereotáxica, a qual é amplamente descrita na literatura cientifica corrente (ENGIN & TREIT, 2007; TSENOV et al., 2007; ZUGNO et al., 2007). Nesta técnica, as coordenadas relativas e específicas para cada estrutura cerebral baseiam-se no atlas de coordenadas estereotáxicas desenvolvido por Paxinos e Watson (1986). A verificação do posicionamento correto da cânula, para a execução dos experimentos desenvolvidos ao longo deste trabalho, foi realizada através da infusão intra-estriatal de azul de metileno, sendo descartados os animais que apresentassem alterações na localização da cânula. Além disso, em todos os experimentos, as infusões intra-estriatais da droga ocorreram dois dias após a cirurgia, a fim de evitar alguma influência das substâncias anestésicas nos resultados obtidos, uma vez que estudos apontam efeito neuroprotetor para a xilazina e ketamina (OZDEN e ISENMANN, 2004). Para tanto, uma agulha (0,9 mm) para a infusão da droga foi adaptada no interior da cânula guia colocada no animal e 2 μL de uma solução de hipoxantina (20 pmol/ 2μl) ou veículo (salina) foram administrados no estriado direito do animal por um intervalo de 1 mim. A dose de hipoxantina utilizada em nosso estudo foi escolhida de acordo com Puig e colaboradores (1989), semelhantes às encontradas em pacientes com Lesch Nyhan. O estriado direito foi utilizado como local para a administração da droga devido às evidentes lesões estriatais encontradas nos pacientes portadores da doença de Lesch Nyhan (VISSER at al., 2000, JINNAH & FRIEDMANN, 2001). Os animais onde foram evidenciadas alterações na posição da cânula não foram utilizados durante a fase experimental. Este modelo foi utilizado ao longo de todos os experimentos descritos nesse trabalho.

Capítulo I – Artigo 01

Intrastriatal hypoxanthine reduces Na^+, K^+ - ATPase activity and induces oxidative stress in the rats

Caren Serra Bavaresco, Fabria Chiarani, Clovis Milton Duval Wannmacher, Carlos Alexandre Netto and Angela Terezinha de Souza Wyse

Publicado na revista Metabolic Brain Disease, 2007.

Intrastriatal hypoxanthine reduces Na^+,K^+ - ATPase activity and induces oxidative stress in the rats

Caren Serra Bavaresco, Fabria Chiarani, Clovis Milton Duval Wannmacher, Carlos Alexandre Netto and Angela Terezinha de Souza Wyse[1]

Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil

[1]Address reprint request to: Dr. Angela T.S. Wyse, Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2600-Anexo, CEP 90035-003 Porto Alegre, RS, Brazil, Telephone number: 55 51 33165573; E-mail: wyse@ufrgs.br

Acknowledgements

This work was supported in part by grants from CNPq – Brazil.

Abstract

The main objective of this study was to investigate the effects of a single intrastriatal injection of hypoxanthine, a metabolite accumulated in Lesch Nyhan diseaseand possibly involved in its neuropathology, on Na^+,K^+-ATPase activity, as well as on some parameters of oxidative stress, namely chemiluminescence (an index of lipid peroxidation), total radical-trapping antioxidant parameter - TRAP (an index of total antioxidant capacity of the tissue) and total thiol protein membrane content, in striatum, cerebral cortex and hippocampus of rats. Results show that hypoxanthine significantly decreased Na^+,K^+-ATPase activity and TRAP while increased chemiluminescence in all ipsislateral structures tested. However, no effect on total thiol protein membrane content was detected. We suggest that hypoxanthine induces oxidative stress in all cerebral structures studied (striatum, hippocampus and cerebral cortex) and that the reduction of Na^+,K^+-ATPase activity was probably mediated by reactive oxygen species.

Key Words: Na^+,K^+-ATPase; Lesch-Nyhan; metabolic disease; hypoxanthine; intrastriatal injection; oxidative stress.

1. Introduction

Tissue accumulation of hypoxanthine occurs in Lesch Nyhan disease, an metabolic disorder caused by severe deficiency of hypoxanthine-guanine phosphoribosyltranspherase activity (Nyhan *et al.*, 1965; Rijksen *et al*., 1981; Harkness *et al*., 1988; Jinnah and Friedmann, 2001). Mental retardation and self-mutilation behavior, characterized by biting of the lips, tongue and fingers with apparent tissue loss, are symptoms of affected patients by this disease (Jinnah *et al.*, 1990; Jinnah and Friedmann, 2001). In addition, they also present dysfunction of the dopamine transmitter system of the basal ganglia (Jinnah and Friedmann, 2001).

Although the underlying mechanisms of brain dysfunction in Lesch-Nyhan disease are poorly understood, the accumulation of oxypurines, such as hypoxanthine, has been proposed to contribute to the neurological dysfunction present (Dasheiff, 1980; Kisch *et al.,* 1985; Visser and Jinnah, 2000; Ma *et al*., 2001). Additionally, hypoxanthine has been implicated in other neurological diseases, including hydrocephalus (Schimidt *et al*., 1995) and cerebral ischemia (Hagberg *et al*., 1987; Palmer, 1987).

Na^+, K^+ - ATPase (EC 3.6.1.37) is an enzyme embedded in the cell membrane, responsible for the generation of the membrane potential through the active transport of sodium and potassium ions in the CNS necessary to maintain neuronal excitability. It is present at high concentrations in brain, consuming 50% of the ATP generated in this tissue (Erecinska and Silver, 1994). It has been demonstrated that Na^+, K^+ - ATPase activity is decreased in epilepsy (Grisar, 1992), in many neurodegenaerative disorders, including Alzheimer´s disease (Lees, 1993; Hattori *et al*., 1998), as well as in experimental cerebral ischemia (Wyse *et al*., 2000); additionally, this enzyme is

inhibited by free radicals (Lees, 1993; Jamme *et al*., 1995). In this context, Na^+, K^+ - ATPase seems to be particularly sensitive to free radical-induced damage since its activity has been associated with plasma membrane lipid composition (Jamme *et al*., 1995) and the redox state of sulfhydryl groups (Morel *et al*., 1998).

We have previously demonstrated that hypoxanthine, xanthine and uric acid significantly inhibit Na^+,K^+-ATPase activity from purified synaptic plasma membrane, suggesting a direct action of these compounds on the enzyme (Bavaresco *et al*., 2004). However, a recent work shows that the inhibition of Na^+,K^+-ATPase activity after preincubation of rat striatum homogenates with hypoxanthine is prevented by trolox and glutathione and was not affected by allopurinol, an inhibitor of xanthine oxidase (Bavaresco *et al*., 2005); that suggests the enzyme inhibition by the oxypurine may have been mediated by lipid peroxidation and sulfhydryl groups oxidation.

In the present study we investigated the *in vivo* effect of intrastriatal injection of hypoxanthine on Na^+,K^+-ATPase activity from synaptic plasma membrane in rat striatum, cerebral cortex and hippocampus, structures involved in motor control and cognition. We also tested the effect of hypoxanthine on some parameters of oxidative stress namely chemiluminescence, total radical-trapping antioxidant parameter (TRAP) and total thiol protein membrane content in the same cerebral structures. Our hypothesis is that intrastriatal hypoxanthine would produce oxidative stress and decrease Na^+,K^+-ATPase in rat brain. The drug was infused in striatum because patients with this syndrome present characteristic alterations in the basal ganglia (Jinnah and Friedmann, 2001).

2. Experimental Procedures

2.1. Animals and reagents

Eighty Wistar rats with 60 days of age (180-200g) were obtained from the Central Animal House of the Department of Biochemistry, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil. Animals were maintained on 12h /12h light/dark cycle (lights on from 7 a.m. to 7 p.m.) in an air-conditioned constant temperature (22^{o} C) colony room and had free access to a 20% (w/w) protein commercial chow and water. Animal care followed the official governmental guidelines in compliance with the Federation of Brazilian Society for Experimental Biology and was approved by the Ethics Committee of the Federal University of Rio Grande do Sul, Brazil. All chemicals were purchased from Sigma Chemical Co., St Louis, MO, USA.

2.2. Stereotaxic Surgery and placement of cannula

Rats were anesthetized with ketamine and xilazine (75 and 10 mg/kg i.p., respectively) and placed in a rodent stereotaxic apparatus. Under stereotaxic guidance, a 27-gauge staineless cannula (0.9 mm O.D.) with an inner needle guide was inserted unilaterally into the right striatum (coordinates relative from bregma: AP, -0.5 mm; ML -2.5 mm; V -2.5 mm from the dura) (Paxinos and Watson, 1986). The cannulae were fixed to the scull with dental cement. Two days after the surgery, a 30–gauge needle was inserted into the guide cannula in order to inject 2 μL buffered hypoxanthine (10 μM) or vehicle (saline) into the right striatum over a 1 min interval. Animals were divided into three groups: group 1 (control group), rats that did not undergo surgery; group 2 (sham group), rats that received intrastriatal vehicle (saline) and group 3 (hypoxanthine treated), rats that received intrastriatal hypoxanthine solution (10 μM). The volume administered (saline or hypoxanthine) was 2 μL; hypoxanthine concentration was chosen according to Puig and colleagues (1989). Rats were sacrificed 30 min after

hypoxanthine or vehicle (saline) injections.

2.3. Tissue preparation

Animals were killed by decapitation without anesthesia, the brain was removed and cerebral structures - striatum, cerebral cortex and hippocampus - were dissected out.

For Na^+, K^+-ATPase activity determination, all cerebral structures were homogenized in 10 volumes 0.32 mM sucrose solution containing 5.0 mM HEPES (pH 7.45) and 1.0 mM EDTA, pH 7.4 (Wyse *et al*., 2000)

For TRAP and chemiluminescence determinations, cerebral structures were homogenized in 10 volumes (1:10) of 0.1 M glycine buffer, pH 8.6 or in 10 volumes (1:10, w/v) of 20.0 mM phosphate buffer containing 140.0 mM KCl, pH 7.4, respectively (Wyse *et al*., 1999).

For membrane protein thiol content determination, tissues were homogenized in phosphate buffered saline (PBS, pH 7.5). Samples were centrifuged at 1.000 X g for 10 min and the supernatants were used for assay determination (Aksenov and Markesbery, 2001).

2.3. Preparation of synaptic plasma membrane striatum, cerebral cortex and hippocampus

Synaptic plasma membranes were prepared according to the method of Jones and Matus (1974), with some modifications (Wyse *et al*., 2000). Striatal, cerebral cortex and hippocampus homogenates were centrifuged at 1,000 g for 20 min and the supernatant removed and centrifuged at 12,000 x g for a further 20 min. The pellet was then resuspended in hypotonic buffer (5.0 mM Tris-HCl buffer, pH 8.1), incubated at 0^0C for 30 min and applied on a discontinuous sucrose density gradient consisting of successive

layers of 0.3, 0.8 and 1.0 M. After centrifugation at 69,000 x *g* for 2 h, the fraction at the 0.8–1.0 M sucrose interface was taken as the membrane enzyme preparation.

2.4. *Determination of Na^+, K^+ - ATPase activity*

The reaction mixture for the Na^+,K^+-ATPase assay contained 5.0 mM $MgCl_2$, 80.0 mM NaCl, 20.0 mM KCl, and 40.0 mM Tris-HCl buffer, pH 7.4, in a final volume of 200 μL. The reaction was started by the addition of ATP (disodium salt, vanadium free) to a final concentration of 3.0 mM. Controls were assayed under the same conditions with the addition of 1.0 mM ouabain. Na^+,K^+-ATPase activity was calculated by the difference between the two assays as described by Wyse and colleagues (2000). Released inorganic phosphate (Pi) was measured by the method of Chan and colleagues (1986); enzyme specific activity was expressed as nmol Pi released per min per mg of protein.

2.5. *Total radical-trapping antioxidant parameter (TRAP) assay*

TRAP assay measures the total antioxidant capacity of the tissue and was determined by measuring the luminol chemiluninescence intensity induced by 2,2´-Azo-bis (2-amidinopropane) (ABAP), according to the method of Lissi and colleagues (1992). Briefly, 4.0 ml of 10.0 mM ABAP were added to the vial and background chemiluminescence was measured. Ten μl of 0.2 μM trolox or homogenates (1:10 in 0.1 M glycine buffer, pH 8.6) were added and chemiluminescence was measured until it reached the initial levels. The addition of trolox or tissue homogenate to the incubation medium reduces the chemiluminescence. The time necessary for return to the levels present before the addition was considered to be the induction time (IT). IT is directly

proportional to the antioxidant capacity of the tissue and was compared to IT of trolox. Results were represented as nM trolox / mg protein.

2.6. *Chemiluminescence assay*

Chemiluminescence is an index of oxidative stress that quantifies lipid peroxidation and was measured according to Gonzalez–Flecha and colleagues (1991). This method is based on the measurement of light emitted (chemluminescence) when the excited carbonyl and singlet oxygen produced by peroxyl radicals decay to ground state. This light represents the generation of reactive oxygen species in whole organs. The entire procedure was carried out in a dark room using a beta liquid scintillation spectrometer (Tricarb 2100). The incubation medium consisted of 20 mM sodium phosphate, pH 7.4, containing 140 mM KCl. A 3.5 ml aliquot of this incubation was added to scintillation vials and chemiluminescence was measured for 2 min. The last measurement was considered as the background chemiluminescence of each vial. Samples were assayed one by one at room temperature. Tissues homogenates (500 µl) were added to the incubation medium, the chemiluninescence was measured for 20 min and the background chemiluminescence was subtracted from the final value. Chemiluninescence was calculated as cpm/mg protein.

2.7. Total thiol protein membrane content:

Membrane protein thiol content was determined using the 5,5'-dithiobis (2-nitrobenzoic acid) method (DTNB), as described by Aksenov and Markesbery (2001) with some modification. Briefly, 50 µl of the sample was mixed with 980 µl of phosphate buffer saline (PBS), pH 7.5, containing 1 mM EDTA. The reaction was started by the addition of 30 µl of 10 mM DTNB stock solution in PBS. After 30 min of

incubation at room temperature, the absorbance at 412 nm was measured and the amount of DTNB formed was calculated. Data were adjusted by protein content.

2.8. *Protein determination*

Protein was measured by the method of Lowry *et al.* (1951) or Bradford (1976) using bovine serum albumin as standard.

2.9. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) followed by the Duncan´s multiple range test when the F-test was significant. All analyses were performed using the Statistical Package for the Social Science (SPSS) software in a PC-compatible computer. A value of $p<0.05$ was considered to be significant.

3. Results

Figure 1 shows the effect of intrastriatal injection of hypoxanthine on Na^+, K^+ - ATPase activity. Hypoxanthine significantly inhibited Na^+, K^+ - ATPase activity in ipsislateral striatum (51%) [$F(2,9)=22.157$; $p<0.01$] (A), cerebral cortex (44%) [$F(2,9)=21.378$; $p<0.01$] (B) and hippocampus (40%) [$F(2,9)=9.984$; $p<0.01$] (C), when compared to naive and sham groups. Enzyme activity in the contralateral striatum, cerebral cortex and hippocampus were not altered (data not shown).

Next, we evaluated the effect of intrastriatal hypoxanthine on some parameters of oxidative stress namely chemiluminescence and TRAP. Figure 2 shows that hypoxanthine significantly increased chemiluminescence in ipsislateral striatum (30%) [$F(2,9)=8.93$; $p<0.01$] (A), cerebral cortex (40%) [$F(2,9)=7.44$; $p<0.01$] (B) and

hippocampus (27%) [$F(2,9)=8.76$; $p<0.01$] (C). Conversely, as shown in figure 3, hypoxanthine decreased TRAP in striatum (51%) [$F(2,9)=16.12$; $p<0.01$] (A), cerebral cortex (36%) [$F(2,9)=6.70$; $p<0.01$] (B) and hippocampus (57%) [$F(2,9)=34.68$; $p<0.01$] (C) when compared to naive and sham groups.

We also verified that intrastriatal hypoxanthine did not alter total thiol protein membrane (Table 1) in any of the structures studied (striatum [$F(2,15)=0.43$, $p>0.05$]; cerebral cortex [$F(2,15)=1.73$ $p>0.05$] and hippocampus [$F(2,15)=0.66$, $p>0.05$], when compared to naive and sham group.

4. Discussion

In the present study we demonstrated that intrastriatal injection of hypoxanthine significantly reduced Na^+, K^+-ATPase activity in synaptic plasma membrane of rat striatum (51%), cerebral cortex (44%) and hippocampus (40%). These results are in agreement with our previous findings, showing that hypoxanthine *in vitro* inhibited Na^+, K^+-ATPase activity in striatum of rats with 6 days of age (Bavaresco *et al*., 2004) and that the decrease of enzyme activity in synaptic plasma membrane from homogenates preincubated with this oxypurine involves oxidative stress (Bavaresco *et al*., 2005).

The inhibition of Na^+, K^+-ATPase activity has been associated with excitotoxicity (Golden *et al.*, 2001); it has been demonstrated that the impairment of the enzyme activity may lend striatal neurons more sensitive to the neurotoxicity of glutamate (Calabresi *et al*., 1983). Other studies suggest that Na^+, K^+-ATPase inhibition is involved in spongiform encephalopathies and in convulsive status (Renkawek *et al*., 1992; Calandriello *et al*., 1995). Additionally, studies demonstrated that neuronal death associated with a decrease in Na^+, K^+ - ATPase activity is mediated by intracellular

depletion of K^+ and accumulation of Ca^{2+} and Na^+ (Xiao *et al*., 2002). In this context, Yang and colleagues (1992) demonstrated that reduction on Na^+, K^+ - ATPase activity resultsin disruption of cellular ionic homeostasis and may be linked with development of cytotoxic brain edema after permanent occlusion of middle cerebral artery.

Interestingly, striatum, cerebral cortex and hippocampus constitute an interconnected neural network (Mahon *et al*. 2004) which may explain the fact that intrastriatal hypoxanthine administration inhibited Na^+,K^+-ATPase in all three structures studied. In this context, Wooten and Collins (1980) demonstrated that intrastriatal injection of kainic acid exhibited a dose-dependent increase in glucose utilization in ipsislateral regions, including hippocampus, pyriform cortex, entorhinal cortex and amygdaloid nuclei. Besides this, intrastriatal kainic acid also provokes local and distant neuronal degeneration in structures such as pyriform cortex, amygdala and deep layers of the cerebral cortex (Zaczek *et al*. 1980). However, we can not discard the possibility that hypoxanthine spread to hippocampus and cortex and therefore directly inhibiting Na^+,K^+-ATPase activity.

We also investigated the effects of intrastriatal hypoxanthine administration into right striatum of rats on some parameters of oxidative stress namely TRAP, chemiluminescence and total thiol protein membrane content. Our results showed that hypoxanthine significantly increased chemiluminescence and decreased TRAP, indicating that peroxidation of membrane lipids and/or reduction on antioxidant defenses may be associated to inhibition of enzyme caused by hypoxanthine. On the other hand, we also observed that intrastriatal hypoxanthine administration did not alter total thiol protein membrane content, what may suggest that, after 30 min of hypoxanthine administration, SH-groups modifications are not involved in inhibition of Na^+, K^+-ATPase activity.

Oxidative stress has been widely implicated in pathogenesis of several neurodegenarative disorders (Halliwell, 2002; Zarkovic, 2003; Behl, 2005). It has been that the brain is highly susceptible to oxidative stress because it has low cerebral antioxidant defenses compared to other tissues (Halliwell, 1996; Floyd, 1999), a fact that makes it more vulnerable to increases in reactive oxygen species. Since oxidative stress is defined as the imbalance between free radical production and antioxidant defenses, our results indicate that hypoxanthine induces free radical generation (enhanced chemiluminescence) and reduces the antioxidant defenses (decreased TRAP), i.e., elicits oxidative stress in the brain. These findings are in agreement with those in a knockout mouse model of Lesch-Nyhan disease showing an induction of oxidative stress, particularly lipid peroxidation (Visser *et al.*, 2002). In addition, Chan and colleagues (1984) demonstrated that a mixture of xanthine and xanthine oxidase could cause brain edema, probably mediated by oxidative stress.

Although the exact mechanism through which hypoxanthine inhibits Na^+,K^+-ATPase activity is still unknown, present *in vivo* findings suggest the participation of reactive oxygen species (ROS) and/or lipid peroxidation in the inhibitory action of hypoxanthine on this enzyme. On the other hand, we can not discard a direct binding of hypoxantine to the Na^+,K^+-ATPase versus an indirect inhibition via ROS production since these processes could be occur in parallel. Considering that we previously showed that hypoxanthine inhibits Na^+,K^+-ATPase activity *in vitro* (direct effect), it is possible that initially this oxypurine inhibits directly the activity of enzyme, leading to an increase in ROS production that in turn feeds back on the enzyme. In this context, it has been shown that Na^+,K^+-ATPase inhibition increase mitochondrial ROS production in cultured cardiac myocytes (Xie *et al*., 1999; Xie and Cai, 2003).

Although it is difficult to extrapolate our results to the human condition, it is conceivable that the inhibition of ectonucleotidases activities in rat striatum might be involved in the pathophysiology of the neurological features present in patients with Lesch-Nyhan disease. However, more studies are necessary to investigate other mechanisms involved in this metabolic condition.

References

Aksenov, M.Y., Markesbery, W.R. (2001). Changes in thiol content and expression of glutathione redox system genes in hippocampus and cerebellum in Alzheimer ´s disease. *Neurosci. Lett*. **302:** 141 – 145.

Bavaresco, C.S., Zugno, A. I., Tagliari, B., Wannmacher, C.M.D., Wajner, M., Wyse, A.T.S. (2004). Inhibition of Na^+, K^+ - ATPase activity in rat striatum by metabolites accumulated in Lesch Nyhan disease. *Int. J. Devl. Neurosci*. **22:** 11 -17.

Bavaresco, C.S., Chiarani, F., Matté, C., Wajner, M., Netto, C.A., Wyse, A.T.S. (2005). Effect of hypoxanthine on Na^+,K^+-ATPase activity and some parameters of oxidative stress in rat striatum. *Brain Res.* **1041:** 198 - 204.

Behl, C. (2005). Oxidative stress in Alzheimer's disease: implications for prevention and therapy. *Subcell Biochem*. **38:** 65-78.

Bradford, M.M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein –die binding. *Anal. Biochem.* **72:** 248 – 254.

Calabresi, P., De Murtas, M., Pisani, A., Stefani, A., Sancessario, G., Mercuri, N.B., Bernardi, G. (1995). Vulnerability of medium spiny striatal neurons to glutamate: role of Na^+,K^+-ATPase activity. *Eur. J. Neurosci*. **7:** 1674 -1683.

Calandriello, L., Curini, R., Pennisi, E.M., Palladini, G. (1995). Spongy state (status spongiosus) and inhibition of Na^+, K^+ - ATPase: a pathogenetic theory. *Med. Hypotheses*. **44:** 173 – 178.

Chan, P.H., Schmidley, J.W., Fishman, R.A., Longar, S.M. (1984). Brain injury, edema and vascular permeability changes induced by oxygen derived free radicals. *Neurology* **34:** 315 - 320.

Chan, K.M., Delfert, D., Junger, K.D. (1986). A direct colorimetric assay for Ca^+ -stimulated ATPase activity. *Anal. Biochem.* **157:** 375 - 380.

Dasheiff, R.M. (1980). Benzodiazepinic treatment for Lesch-Nyhan syndrome? *Dev. Med. Child. Neurol.* **22:** 101 - 102.

Erecinska, M., Silver, I.A. (1994). Ions and energy in mammalian brain. *Prog. Neurobiol.* **7:** 21 - 29.

Floyd, R.A. (1999). Antioxidants, oxidative stress and degenerative neurological disorders. *Proc. Soc. Exp. Biol. Med*. **222:** 236 - 245.

Golden, W.C., Brambrink, A.M., Traystman, R.J., Martin, L.J. (2001). Failure to sustain recovery of Na^+,K^+-ATPase function is a possible mechanism for striatal neurodegeneration in hypoxic-ischemic newborn piglets. *Brain Res. Mol. Brain Res.* **81:** 94 - 102.

Gonzalez Flecha, B., Llesuy, S., Boveris, A. (1991). Hydroperoxide-initiated chemiluminescence: an assay for oxidative stress in biopsies of heart, liver, and muscle. *Free Radic. Biol. Med.* **10:** 93-100.

Grisar, T. (1984). Glial and neuronal Na^+,K^+-pump in epilepsy. *Ann. Neurol.* **16 (Suppl.):** 128 - 134.

Hagberg, H., Andersson, P., Lacarewicz, J., Jacobson, I., Butcher, S., Sandberg, M. (1987). Extracellular adenosine, inosine, hypoxanthine and xanthine to tissue nucleotides and purines in rat striatum during transient ischemia. *J. Neurochem.* **49:** 227 - 231.

Halliwell, B. (1996). Free radicals, proteins and DNA: oxidative damage versus redox regulation. *Biochem. Soc. Trans.* **24:** 1023 - 1027.

Halliwell, B. (2002). Hypothesis: proteasomal dysfunction: a primary event in neurogeneration that leads to nitrative and oxidative stress and subsequent cell death. *Ann. N. Y. Acad. Sci.* **962:** 182 - 94.

Harkness, R.A., McCreanor, G.M., Watts, R.W. (1988). Lesch-Nyhan syndrome and its pathogenesis: purine concentrations in plasma and in urine with metabolite profiles in CSF. *J. Inher. Metab. Dis.* **11:** 239 - 252.

Hattori, N., Kitagawa, K., Higashida, T., Yagyu, K., Shimohama, S., Wataya, T., Perry, G., Smith, M.A., Inagaki, C. (1998). Cl^- - ATPase and Na^+/K^+ - ATPase activities in Alzheimer´s disease brains. *Neurosci. Lett.* **254:** 141 - 144.

Jamme, I., Petit, E., Divoux, D., Gerbi, A., Maixent, J.M., Nouvelot, A. (1995). Modulation of mouse cerebral Na^+,K^+-ATPase activity by oxygen free radicals. *Neuroreport* **7:** 333 - 337.

Jinnah, H.A., Friedmann, T. (2001). Lesch Nyhan disease and it variants. In: Scriver, C.R., Beaudet, A.L., Sly, W.S., Valle, D. (Eds.), *The Metabolic and Molecular Bases of Inherited Disease, eighth ed.* McGraw-Hill, New York, pp. 2537-2569.

Jinnah, H.A., Gage, F.H., Friedmann, T. (1990). Animal models of Lesch-Nyhan syndrome. *Brain Res. Bull.* **25:** 467 - 475.

Jones, D.H., Matus, A.I. (1974). Isolation of synaptic plasma membrane from brain by combined flotation-sedimentation density gradient centrifugation. *Biochim. Biophys. Acta.* **356:** 276 - 287.

Kisch, S.J., Fox, I.H., Kapur, B.M., Lloyd, K.G., Hornykiewicz, O. (1985). Brain benzodiazepine receptor binding and purine concentration in Lesch-Nyhan. *Brain Res.* **336:** 117-123.

Lees, G.J. (1993). Contributory mechanism in the causation of neurodegenerative disorders. *Neurosci.* **54:** 287 - 322.

Lissi, E., Pascual, C., Del Castillo, M.D. (1992). Luminol luminescence induced by 2,2' –azo-bis (2-amidinopropane) thermolysis. *Free Rad. Res. Comm.* **17:** 299 - 311.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265 – 267.

Ma, M.H.Y., Stacey, N.C., Connolly, G.P. (2001). Hypoxanthine impairs morphogenesis and enhances proliferation of a neuroblastoma model of Lesch Nyhan syndrome. *J. Neurosci. Res.* **63:** 500 - 508.

Mahon, S., Deniau, J.M., Charpier. S. (2004). Corticostriatal plasticity: life after the depression. *Trends Neurosci.* **27:** 460 – 467.

Morel, P., Faucommeau, B., Page, G. (1998). Inhibitory effect of ascorbic acid on dopamine uptake by rat striatal synaptosomes: relationship to lipid peroxidation and oxidation of protein sulfhydryl groups. *Neurosci. Res.* **32:** 171 – 179.

Nyhan, W.L., Oliver, W.J., Lesch, M. (1965). A familial disorder or uric acid metabolism and central nervous system function II. *J. Pediatr.* **67:** 439 -444.

Palmer. G.C. (1987). Free radicals generated by xanthine oxidase – hypoxanthine damage adenylate cyclase and ATPase in gerbil cerebral cortex. *Metab. Brain. Dis.* **2:** 243 – 257.

Paxinos, G., Watson, C. (1986). *The rat brain in stereotaxic coordinates*, second ed. Academic Press, London.

Puig, J.G., Jimenez, M.L., Mateos, F.A., Fox, I.H. (1989). Adenine nucleotide turnover in hypoxanthine-guanine phosphoribosyl-transferase: evidence for an increased contribution of purine biosyntheses de novo. *Metabolism* **38:** 410 - 418.

Renkawek, K., Renier, W.O., de Pont, J.J., Vogels, O.J., Gabreels, F.J. (1992). Neonatal status convulsivus, spongioform encephalopathy, and low activity of Na^+, K^+ - ATPase in the brain. *Epilepsia* **33:** 58 – 64.

Rijksen, G., Staal, G.E.J., Van der Vlist, M.J.M., Beerner, F.A., Troost, J., Gutensohn, W., Van Laarhoven, J.P.R.M., De Bruyn, C.H.M.M. (1981). Partial hypoxanthine-guanine phosphoribosyl transferase deficiency with full expression of the Lesch – Nyhan syndrome. *Hum. Gen.* **57:** 39 - 47.

Schmidt, H., Siems, W.G., Grune, T., Grauel, E.L. (1995). Concentration of purine compounds in the cerebrospinal fluid of infants suffering from sepsis, convulsions and hydrocephalus. *J. Perinat. Med.* **23:** 167 -174.

Visser, J.E., Bär, P.R., Jinnah, H.A. (2000). Lesch-Nyhan disease and the basal ganglia. *Brain Res. Bull.* **32:** 449 - 475.

Visser, J.E., Smith, D.W., Moy, S.S., Breese, G.R., Friedmann, T., Rothstein, J.D., Jinnah, H.A. (2002). Oxidative stress and dopamine deficiency in a genetic mouse model of Lesch-Nyhan disease. *Brain Res. Dev. Brain Res.* **133:** 127-139.

Wooten, G.F., Collins, R.C. (1980). Regional glicose utilization following intrastriatal injection of kainic acid. *Brain Res.* **201:** 173 – 184.

Wyse, A.T.S., Noriler, M.E., Borges, L.F., Floriano, P.J., Silva, C.G., Wajner, M., Wannmacher, C.M.D. (1999). Alanine prevents the decrease of Na^+,K^+-ATPase activity in experimental phenylketonuria. *Metab Brain Dis.* **14:** 95 -101.

Wyse, A.T.S., Streck, E.L., Worm, P., Wajner, M., Ritter, F., Netto, C.A. (2000). Preconditioning prevents the inhibition of Na^+,K^+-ATPase activity after brain ischemia. *Neurochem. Res.* **25:** 969 - 973.

Xie, Z., Kometiani, P., Liu, J., Li, J., Shapiro, J.I., Askari, A. (1999). Intracellular reactive oxygen species mediate the linkage of Na^+ -K^+ –ATPase to hypertrophy and its marker genes in cardiac myocytes. *J. Biol. Chem.* **274:** 19323–19328.

Xie, Z., Cai, T. (2003). Na^+,K^+-ATPase – mediated signal transduction: from protein interaction to cellular function. *Molecular Interventions* **3:** 157-168.

Xiao, A.Y., Wei, L., Xia, S., Rothman, S., Yu, S.P. (2002). Ionic mechanism of ouabain-induced concurrent apoptosis and necrosis in individual cultured cortical neurons. *J. Neurosci.* **22:** 1350 - 1362.

Yang, G.Y., Chen, S.F., Kinouchi, H., Chan, P.H., Weinstein, P.R. (1992). Edema, cation content, and ATPase activity after middle cerebral artery occlusion in rats. *Stroke* **23:** 1331 – 1336.

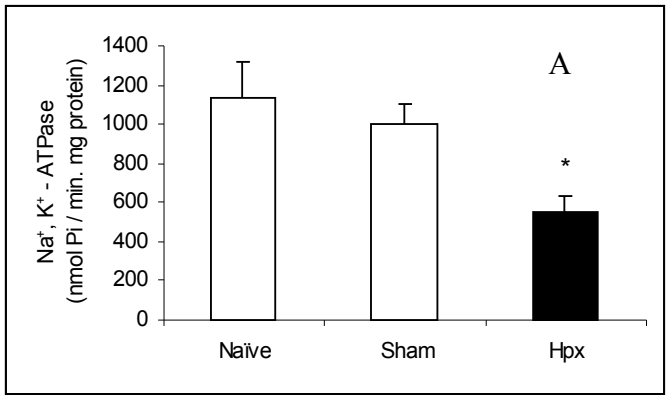
Zaczek, R., Simonton, S., Coyle, J.T. (1980). Local and distant neuronal degeneration following intrastriatal injection of kainic acid. *J. Neuropathol. Exp. Neurol.* **39:** 245 – 264.

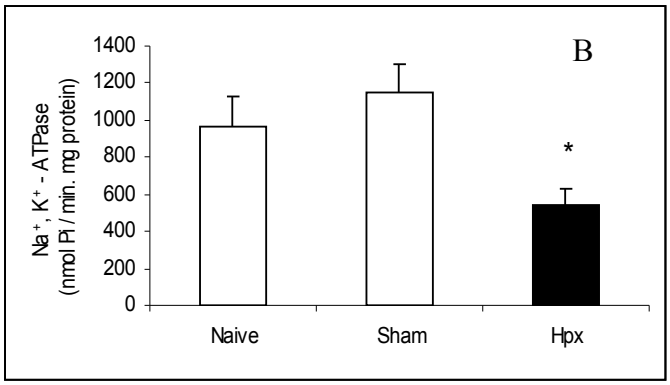
Zarkovic, K. (2003). 4-hydroxynonenal and neurodegenerative diseases. *Mol. Aspects Med.* **24:** 293-303.

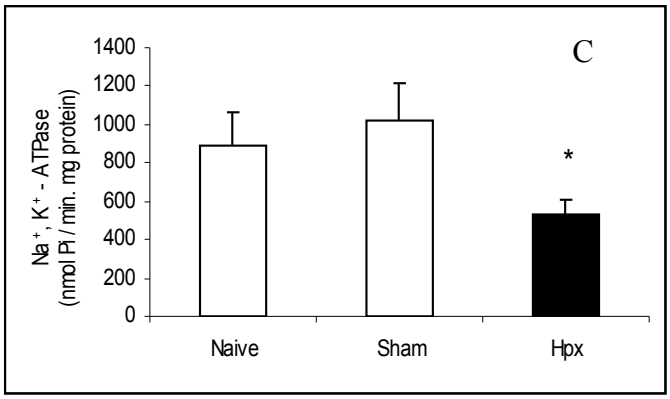
Figure 1. Effect of intrastriatal hypoxanthine injection on Na^+, K^+ - ATPase activity in the synaptic plasma membrane from striatum (A), cerebral cortex (B) and hippocampus (C) of rats. Data are means ± SD for four independent experiments (animals) performed in duplicate. * $p<0.01$ compared to all other groups (Duncan´s multiple range test). Hpx - hypoxanthine

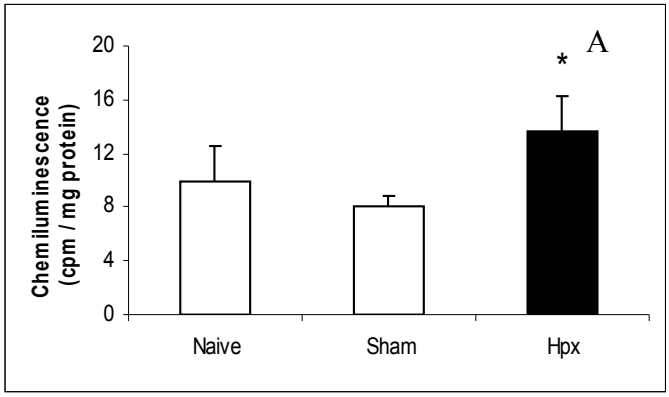
Figure 2.. Effect of intrastriatal hypoxanthine injection on chemiluninescence (an index of lipid peroxidation) in striatum (A), cerebral cortex (B) and hippocampus (C) of rats. Data are means ± SD for four independent experiments (animals) performed in duplicate. * $p<0.01$ compared to all other groups (Duncan´s multiple range test). Hpx – hypoxanthine.

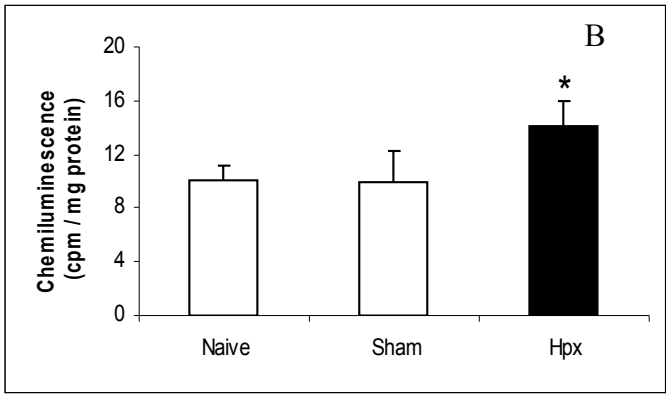
Figure 3. Effect of intrastriatal hypoxanthine injection on TRAP (an index of total antioxidant capacity of the tissue) in striatum (A), cerebral cortex (B) and hippocampus (C) of rats. Data are means ± SD for four independent experiments (animals) performed in duplicate. * $p<0.01$ compared to all other groups (Duncan´s multiple range test). Hpx – hypoxanthine.

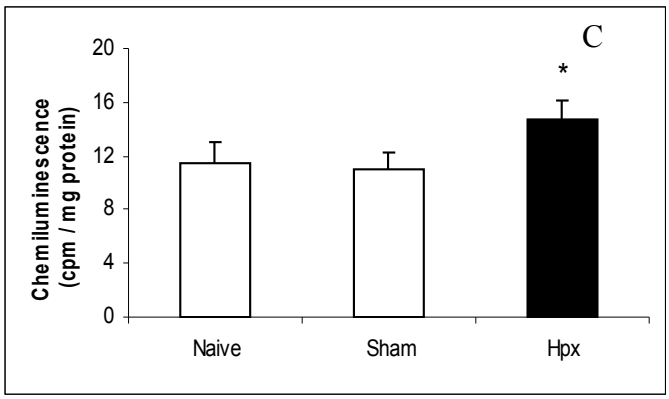

A
1400
1200
1000
800
600
400
200
0
Na+, K+ - ATPase
(nmol Pi / min. mg protein)
*
Naïve
Sham
Hpx

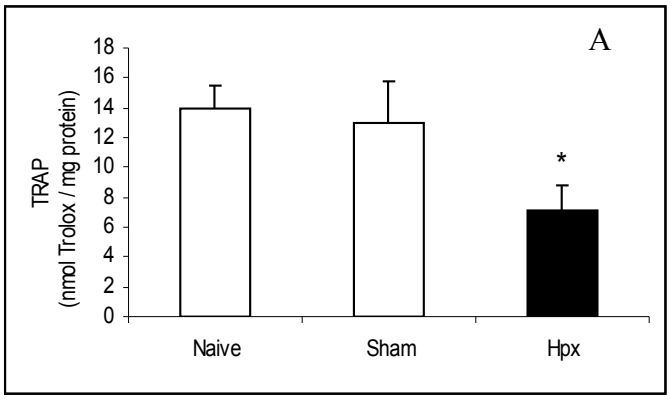

B
1400
1200
1000
800
600
400
200
0
Na+, K+ - ATPase
(nmol Pi / min. mg protein)
*
Naive
Sham
Hpx

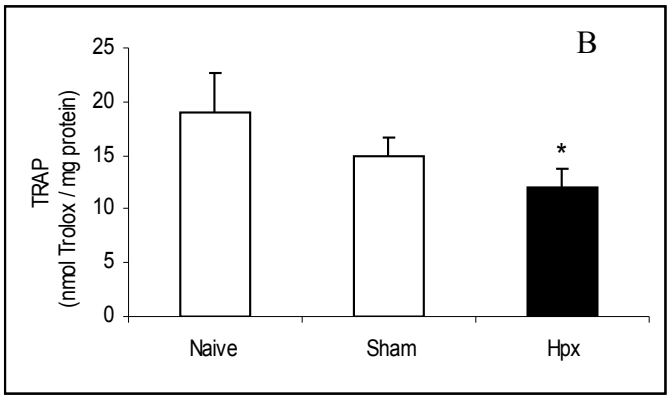

C
1400
1200
1000
800
600
400
200
0
Na+, K+ - ATPase
(nmol Pi / min. mg protein)
*
Naive
Sham
Hpx


A
*
Chemiluminescence (cpm / mg protein)
20
16
12
8
4
0
Naive
Sham
Hpx


B
*
Chemiluminescence (cpm / mg protein)
20
16
12
8
4
0
Naive
Sham
Hpx


C
*
Chemiluminescence (cpm / mg protein)
20
16
12
8
4
0
Naive
Sham
Hpx


A
TRAP
(nmol Trolox / mg protein)
18
16
14
12
10
8
6
4
2
0
*
Naive
Sham
Hpx


B
TRAP
(nmol Trolox / mg protein)
25
20
15
10
5
0
*
Naive
Sham
Hpx

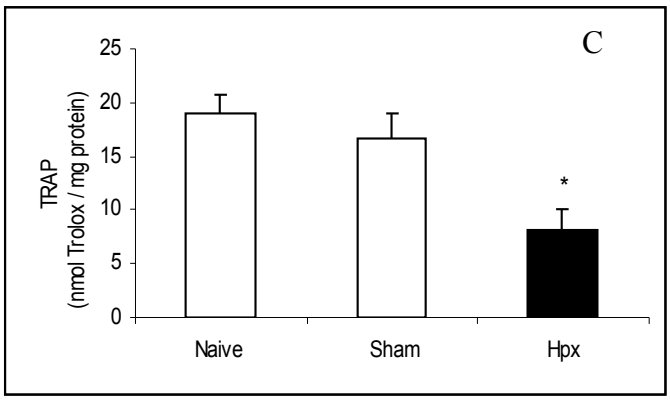

C
TRAP
(nmol Trolox / mg protein)
25
20
15
10
5
0
*
Naive
Sham
Hpx

Table 1. Effect of intrastriatal hypoxanthine injection on total thiol protein membrane content. Data are means ± SD for six independent experiments (animals) performed in duplicate. Results are expressed as nmol TNB/ min. mg protein. Hpx – hypoxanthine.

	Total thiol membrane content (groups)		
Cerebral regions	Naive	Sham	Hpx
Striatum	141.08 ± 14.20	138.40 ± 22.68	137.50 ± 27.40
Hippocampus	154.06 ± 18.40	151.38 ± 32.47	148.25 ± 24.01
Cerebral Cortex	131.00 ± 18.51	156.00 ± 34.71	134.16 ± 19

Capítulo II – Artigo 02

Intrastriatal hypoxanthine administration affects Na^+, K^+ - ATPase, acetylcholinesteraseand catalase activities in striatum, hippocampus and cerebral cortex of rats.

Caren Serra Bavaresco, Fabria Chiarani, Moacir Wajner, Carlos Alexandre Netto and Angela Terezinha de Souza Wyse

Publicado na revista International Journal of Developmental Neuroscience, 2006.

Intrastriatal hypoxanthine administration affects Na^+, K^+ - ATPase, acetylcholinesterase and catalase activities in striatum, hippocampus and cerebral cortex of rats

Caren Serra Bavaresco, Fabria Chiarani, Moacir Wajner, Carlos Alexandre Netto and Angela Terezinha de Souza Wyse[1]

Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil

[1]Address reprint request to: Dr. Angela T.S. Wyse, Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2600-Anexo, CEP 90035-003 Porto Alegre, RS, Brazil, Telephone number: 55 51 33165573; E-mail: wyse@ufrgs.br

Abstract

The aim of this study was to investigate the effects of a single intrastriatal injection of hypoxanthine, the major metabolite accumulating in Lesch Nyhan disease, on Na^+,K^+-ATPase, acetylcholinesterase and catalase activities in striatum, cerebral cortex and hippocampus of rats at different post-infusion periods. Adult Wistar rats were divided in two groups: (1) vehicle-injected group (control) and (2) hypoxanthine-injected group. For Na^+,K^+-ATPase activity determination, the animals were sacrificed 3 h, 24 h and 7 days after drug infusion. For the evaluation of acetylcholinesterase and catalase activities, the animals were sacrificed 30 min, 3 h, 24 h and 7 days after hypoxanthine infusion. Results show that hypoxanthine significantly alters Na^+,K^+-ATPase, acetylcholinesterase and catalase activities. We suggest that these modification on cerebral biochemical parameters (Na^+,K^+-ATPase, acetylcholinesterase and catalase activities) induced by hypoxanthine in all cerebral structures studied, striatum, hippocampus and cerebral cortex, could be involved in the pathophysiology of Lesch Nyhan disease.

Key Words: Lesch-Nyhan; metabolic disease; hypoxanthine; intrastriatal injection; Na^+,K^+-ATPase; acetylcholinesterase; catalase.

1. Introduction

Hypoxanthine is the main purine nucleobase involved in the salvage purine pathway in the brain, being basal ganglia particularly dependent of this route to maintain normal tecidual purine levels (Jinnah and Friedmann, 2001). Severe alteration in this pathway, due to deficiency on hypoxanthine-guanine phosphoribosyltranspherase activity causes Lesch Nyhan disease (Nyhan et al., 1965; Herderson et al., 1968; Rijksen et al., 1981; Jinnah and Friedmann, 2001). Affected patients present hyperuricemia, spasticity, dystonia, cognitive deficits and self-mutilation behavior, which is characterized by biting of the lips, tongue and fingers with apparent tissue loss (Mizuno, 1986; Jinnah and Gage, 1990; Matthews et al., 1999; Jinnah and Friedmann, 2001). In addition, patients present a prominent loss of striatal dopamine (Jinnah and Friedmann, 2001). A common factor in Lesch Nyhan is that patients exhibit a characteristically raised level of hypoxanthine in urine (Harkness et al., 1988), plasma and cerebrospinal fluid (Rosenbloom et al., 1967; Harkness et al., 1988; Puig and Mateos, 1993). Although the underlying mechanisms of brain dysfunction in Lesch-Nyhan are poorly understood, the accumulation of oxypurines such as hypoxanthine has been proposed to contribute to the neurological dysfunction present in this disease (Dasheiff, 1980; Kisch et al., 1985; Visser et al., 2000; Ma et al., 2001).

Na^+, K^+ - ATPase (EC 3.6.1.37) is an enzyme embedded in the cell membrane, responsible for the generation of the membrane potential through the active transport of sodium and potassium ions in the central nervous system necessary to maintain neuronal excitability. It is present at high concentrations in brain and consumes 40 – 50% of the ATP generated in this tissue (Erecinska and Silver, 1994). It has been demonstrated that the enzyme activity is inhibited in brain ischaemia (Wyse at al., 2000), epilepsy (Grisar,

1984) and many neurodegenerative disorders (Lees, 1993). Additionally, Na^+, K^+ - ATPase is inhibited by free radicals and/or oxidative stress (Jamme et al., 1995). We have also previously demonstrated that 10 μM hypoxanthine added to the incubation medium inhibits Na^+, K^+ - ATPase activity in plasma synaptic membrane of striatum from six-day-old rats, suggesting a direct effect on the enzyme (Bavaresco et al. 2004).

Acetylcholinesterase (AChE) (EC 3.6.1.37), an enzyme involved in the hydrolysis of the neurotransmitter acetylcholine, contributes to the integrity of the synaptic membrane (Grafius et al., 1971). Acetylcholinesterase has been implicated in cholinergic and non-cholinergic actions such as axonal guidance, neurite outgrowth and synaptogenesis (Lassiter et al., 1998). Besides, studies suggest that this enzyme is associated with the pathophysiology of some neurodegenerative diseases (Henderson et al., 1996; Garcia-Alloza et al., 2005). On the hand other, it has been shown that a decrease in cholinergic function was correlated with aggressive behavior in patients with Alzheimer's disease, supporting a role of the cholinergic system in cognitive and non-cognitive disturbances (Garcia-Alloza et al., 2005). In addition, evidence from the literature has demonstrated that patients with Lesch Nyhan disease present low levels of striatal choline acetyltranferase (Lloyd et al., 1981; Saito and Takashima, 2000).

Oxidative stress is defined as an imbalance between formation and removal of free radicals and it is presumed to be involved in many events related to the pathogenesis of neurodegenerative disorders such as multiple esclerosis, epilectic siezures and Parkinson´s disease (Halliwell and Gutteridge, 1985; Beal, 1995). This may be related to the fact that the brain is highly susceptible to oxidative stress, because it has low cerebral antioxidant defenses as compared to other tissues (Haliwell, 1996; Floyd, 1999), a fact that makes this tissue more vulnerable to increases in reactive oxygen species. In this scenario, it has been demonstrated that hypoxanthine/xanthine oxidase acts as a source

of oxidative stress in the vascular system (Oliveira et al, 2001) and might contribute to the destruction of blood-brain barrier observed in ischemic brain tissue (Beckman et al., 1987). This is probably associated to the fact that hypoxanthine is converted to xanthine and uric acid by xanthine oxidase, producing hydrogen peroxide and superoxide, deleterious oxygen-derived free radicals (Horton, 2003). In this context, a previous study from our group demonstrated that hypoxanthine *in vitro* increased TBARS and reduced TRAP in rat striatum, suggesting an induction of oxidative stress (Bavaresco et al., 2005).

In the present study we investigated the hypothesis that intrastriatal administration of hypoxanthine could alter the activities of Na^+,K^+-ATPase, acetylcholinesterase and catalase activities in striatum, cerebral cortex and hippocampus of rats at different periods after drug administration.

4. Experimental Procedures

2.1. Animals and reagents

Wistar rats with 60 days of age (180-200g) were obtained from the Central Animal House of the Department of Biochemistry, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil. Animals were maintained on 12 h light/dark cycle (lights on from 7 a.m. to 7 p.m.) in an air-conditioned constant temperature (22^o C) colony room and had free access to a 20% (w/w) protein commercial chow and water. Animal care followed the official governmental guidelines in compliance with the Federation of Brazilian Society for Experimental Biology; present investigation was approved by the Ethics Committee of the Federal University of Rio Grande do Sul, Brazil. All chemicals were purchased from Sigma Chemical Co., St Louis, MO, USA.

2.2. Stereotaxic Surgery and placement of cannula

Rats were anesthetized with ketamine and xilazine (75 and 10 mg/kg i.p., respectively) and placed in a rodent stereotaxic apparatus. Under stereotaxic guidance, a 27-gauge staineless cannula (0.9 mm O.D.) with an inner needle guide was inserted unilaterally into the right striatum (coordinates relative from bregma: AP, -0.5 mm; ML -2.5 mm; V -2.5 mm from the dura) (Paxinos and Watson, 1986). The cannulae were fixed to the skull with dental cement. Two days after surgery, a 30–gauge needle was inserted into the guide cannula in order to inject 2 μL of buffered hypoxanthine (10 μM) or vehicle (saline) into the right striatum over a 1 min interval. Animals were divided in two groups: group 1 (vehicle treated), rats that received intrastriatal saline and group 2 (hypoxanthine treated), rats that received intrastriatal hypoxanthine solution (0,0002 μmol/ g tissue). Hypoxanthine dose was chosen according to Puig and colleagues (1989). For AChE and CAT activities assays, animals were sacrificed 30 min, 3 h, 24 h or 7 days after drug administration. For Na^+, K^+-ATPase activity determination, rats were sacrificed 3 h, 24 h or 7 days after hypoxanthine or vehicle (saline) injections because the 30 min effect have been previously reported (Bavaresco et al., 2006).

2.3. Tissue preparation

Animals were killed by decapitation without anesthesia, the brain was removed and cerebral structures - striatum, cerebral cortex and hippocampus - were dissected out. For Na^+, K^+-ATPase activity determination, all cerebral structures were homogenized in 10 volumes (w/v) of 0.32 mM sucrose solution containing 5.0 mM HEPES (pH 7.45) and 1.0 mM EDTA, pH 7.4 (Wyse *et al*., 2000). For AChE activity assay, cerebral structures were homogenized in 10 volumes (w/v) of 0.1 mM of potassium phosphate

buffer, pH 7.5 and centrifuged for 10 min at 1000 X g. The supernatant was used for enzymatic AChE analysis (Ellman et al, 1961). For CAT activity assay, cerebral structures were homogenized in 10 volumes (w/v) of 10.0 mM potassium phosphate buffer, pH 7.6 (Aebi, 1984).

2.4. Preparation of synaptic plasma membrane from striatum, cerebral cortex and hippocampus

Synaptic plasma membranes were prepared according to the method of Jones and Matus (1974), with some modifications (Wyse *et al*., 2000). Striatum, cerebral cortex and hippocampus homogenates were centrifuged at 1,000 g for 20 min and the supernatant removed and centrifuged at 12,000 x g for a further 20 min. The pellet was then resuspended in hypotonic buffer (5.0 mM Tris-HCl buffer, pH 8.1), incubated at 0^0C for 30 min and applied on a discontinuous sucrose density gradient consisting of successive layers of 0.3, 0.8 and 1.0 M. After centrifugation at 69,000 x *g* for 2 h, the fraction at the 0.8–1.0 M sucrose interface was taken as the membrane enzyme preparation.

2.5. *Determination of Na^+, K^+ - ATPase activity*

The reaction mixture for the Na^+,K^+-ATPase assay contained 5.0 mM $MgCl_2$, 80.0 mM NaCl, 20.0 mM KCl, and 40.0 mM Tris-HCl buffer, pH 7.4, in a final volume of 200 μL. The reaction was started by the addition of ATP (disodium salt, vanadium free) to a final concentration of 3.0 mM. Controls were assayed under the same conditions with the addition of 1.0 mM ouabain. Na^+,K^+-ATPase activity was calculated by the difference between the two assays as described by Wyse and colleagues (2000). Released inorganic phosphate (Pi) was measured by the method of Chan and colleagues

(1986); enzyme specific activity was expressed as nmol Pi released per min per mg of protein.

2.6. AChE activity assay

Acetylcholinesterase activity was determined according to Ellman et al. (1961), with some modifications (Villescas et al., 1981). Hydrolysis rates were measured at acetylthiocholine (S) concentrations of 0.8 mM in 1ml of assay solutions with 30 mM phosphate buffer, pH 7.5, and 1.0 mM DTNB at 25^{o} C. Fifty microlitres of cerebral structures supernatant were added to the reaction mixture and preincubated for 3 min. The hydrolysis was monitored by the formation of thiolate dianion of DTNB at 412 nm for 2-3 min (intervals of 30 s). Enzyme specific activity was expressed as umol ASCh per h per mg of protein.

2.7. CAT activity assay

CAT activity was assayed by the method of Aebi (1984), which is based on the disappearance of H_2O_2 at 240 nm. One unit of the enzyme is defined as 1 μmol of hydrogen peroxide consumed per minute and the specific activity was reported as units per mg protein.

2.8. *Protein determination*

Protein was measured by the method of Lowry *et al.* (1951) or Bradford (1976) using bovine serum albumin as standard.

2.9. Statistical analysis

Data were analyzed by the Student´s *t* test. All analyses were performed using the Statistical Package for the Social Science (SPSS) software in a PC- compatible computer. A value of $p<0.05$ was considered to be significant.

3. Results

3.1. Experiment 1: Effect of intrastriatal hypoxanthine infusion on Na^+,K^+-ATPase activity.

Figure 1 shows the effect of intrastriatal injection of hypoxanthine on Na^+, K^+ - ATPase activity in striatum, hippocampus and cerebral cortex at different periods after oxypurine administration. Na^+, K^+ - ATPase activity was significantly inhibited in the ipsislateral striatum after 3 h (45%) [$t(6)=6.569$; $p<0.01$], 24 h (52%) [$t(6)=9.635$; $p<0.01$] and 7 days (71%) [$t(6)=9.018$; $p<0.01$] of hypoxanthine administration (Fig. 1A). As can been seen in Fig. 1 B, this enzyme was also inhibited in hippocampus 3h (44%) [$t(6)=7.424$; $p<0.01$], 24h (42%) [$t(6)=11.769$; $p<0.01$] and 7 days (59%) [$t(6)=6.419$; $p<0.01$] after drug infusion. In contrast, hypoxanthine administration did not affect Na^+, K^+-ATPase activity in cerebral cortex at all periods after injection when compared to sham groups {3 h [$t(6)=0.941$; $p>0.05$]; 24 h [$t(6)=0.128$; $p>0.05$] or 7 days [$t(6)=0.846$; $p>0.05$]} (Fig. 1C). Enzyme activity was not altered after hypoxanthine infusion in contralateral striatum, cerebral cortex and hippocampus (data not shown).

3.2. Experiment 2: Effect of intrastriatal hypoxanthine infusion on acetylcholinesterase activity.

Next, we evaluated the effect of intrastriatal hypoxanthine on acetylcholinesterase activity. Figure 2 A shows that acetylcholinesterase was

significantly inhibited in the ipsislateral striatum after 3 h (42%) [t(6)=5.710; p<0.01] and 24 h (20%) [t(6)=3.056; p<0.05], but not 30 min [t(6)=0.589; p>0.05] or 7 days [t(6)=0.223; p>0.05] after hypoxanthine infusion. In contrast, Figure 2 B shows that in cerebral cortex, acetylcholinesterease activity was increased (62%) 24 h [t(6)=3.597; p<0.01] after hypoxanthine infusion, but not after 30 min [t(6)=1.441; p>0.05], 3 h [t(6)=0.945; p>0.05] or 7 days [t(6)=2.135; p>0.05]. On the other hand, hypoxanthine administration did not cause any modification on this enzyme activity in hippocampus (30 min [t(6)=0.108; p>0.05]; 3 h [t(6)=0.508; p>0.05]; 24 h [t(6)=0.157; p>0.05] and 7 days [t(6)=0.528; p>0.05]) (Fig. 2C). Enzyme activity was not altered after hypoxanthine administration in contralateral striatum, cerebral cortex and hippocampus in contralateral striatum, cerebral cortex (data not shown).

3.3. Experiment 3: Effect of intrastriatal hypoxanthine infusion on catalase activity.

We also verified the effect of intrastriatal injection of hypoxanthine on catalase activity. Figure 3 A shows that catalase activity was significantly inhibited in ipsislateral striatum 30 min (25%) [t(6)=5.629; p<0.01] and significantly increased after 7 days (18%) [t(9)=2.510; p<0.05], with no change at 3 h [t(6)=0.829; p>0.05] and 24h [t(6)=0.175; p<0.05] after hypoxanthine infusion. In cerebral cortex (Figure 3 B), catalase activity was significantly inhibited after 30 min (20%) [t(6)=3.039; p<0.05] and 3 h (20%) [t(8)=3.006; p<0.05] but not 24 h [t(7)=1.487; p>0.05] and 7 days [t(7)=1.441; p>0.05] after hypoxanthine infusion. As can be seen in Figure 3 C, enzyme activity in hippocampus was inhibited 24 h (33%) [t(6)=2.426; p<0.05] and 7 days (32%) [t(7)=4.096; p<0.01] but not 30 min [t(6)=0.346; p>0.05] nor 3 h [t(6)=0.913; p>0.05] after drug administration. Enzyme activity was not altered after hypoxanthine

administration in contralateral striatum, cerebral cortex and hippocampus (data not shown).

4. Discussion

Lesch Nyhan is an X-linked recessive disease of purine metabolism (Nyhan et al., 1965; Jinnah and Friedmann, 2001). It is biochemically characterized by deficiency of HPRT activity, resulting in tissue accumulation of hypoxanthine. Patients present neurological and cognitive disabilities, choreoatetosis, spasticity, mental retardation and characteristic neurobehavioral phenotype characterized by compulsive self-mutilation (Jinnah and Friedmann, 2001).

Considering that the pathomechanisms involved in the brain damage of this disease are poorly known, in the present study we investigated the effect of intrastriatal injection of hypoxanthine on Na^+,K^+-ATPase, acetylcholinesterase and catalase activities in striatum, cerebral cortex and hippocampus of rats. The drug was infused into the striatum because patients with this disease present characteristic alterations in the basal ganglia (Jinnah and Friedmann, 2001). Furthermore, we also studied the activity of these enzymes in the cerebral cortex and hippocampus because there are convergences of cortical projections to different parts of the striatum with afferents from allocortical regions, such as hippocampal formation (Heimer et al., 1995). Thus, these cerebral structures are presumed to work as an integrated system and essential for learning/memory, functions that are severely compromised in Lesch Nyhan (Jinnah and Friedman, 2001).

We observed that intrastriatal hypoxanthine administration inhibits Na^+,K^+-ATPase activity in striatum and hippocampus of rats at all periods after injection. In contrast, Na^+,K^+-ATPase activity in cerebral cortex remained unaltered (Figure 1). Our

findings may be possibly related to the distribution of Na^+,K^+-ATPase alpha isoforms in the CNS regions, explaining therefore the differential response of these cerebral structures to hypoxanthine injection. In this context, Hieber and colleagues (1991) showed that alpha 1 mRNA is predominantly expressed in hippocampus and striatum while cerebral cortex is rich in alpha 3 mRNA. Furthermore, since Na^+,K^+-ATPase activity is inhibited by free radical formation and hypoxanthine induces lipid peroxidation and diminishes the non-enzymatic antioxidant defenses (Bavaresco et al., 2005; Bavaresco et al., 2006), it is possible that the inhibitory effect of hypoxanthine on Na^+,K^+-ATPase activity in striatum and hippocampus could be mediated by oxidative stress. In this context, Stark (2005) showed that lipid peroxidation could contribute to the loss of cellular functions through the inactivation of membrane enzymes. The absence of effect of Na^+,K^+-ATPase from cerebral cortex may be hypothetically attributed to a higher susceptibility of the alpha/RNA subunit to the oxidative attack.

Our results also show that acetylcholinesterase activity is inhibited in striatum 3 h and 24 h, but not 30 min and 7 days, after intrastriatal hypoxanthine infusion (Figure 2). In contrast, this enzyme activity enhances in cerebral cortex only 24h after the administration of drug. On the other hand, intrastriatal hypoxanthine infusion did not affect acetylcholinesterase activity in the hippocampus. So far we do not know the exact mechanisms by which hypoxanthine acts on acetylcholinesterase activity.

Considering that previous studies in a knockout mouse model of Lesch – Nyhan disease showed a induction of oxidative stress (Visser et al., 2002) and that *in vitro* studies demonstrated that hypoxanthine increases TBARS (lipid peroxidation) and decreases TRAP (non-enzymatic antioxidant capacity) (Beckman et al., 1987; Bavaresco et al., 2005), in the present study we analysed the effect of intrastriatal hypoxanthine on catalase activity, an important antioxidant enzyme. Our results showed that

hypoxanthine inhibits catalase activity in striatum 30 min after drug infusion, but enhances enzyme activity after 7 days (Figure 3). This may be possibly attributed to a short term inhibition of the enzyme activity and further to a metabolic adaptation involving the synthesis of more protein to overcome the damage oxidative elicited by hypoxanthine administration. In hippocampus, this enzymatic activity was inhibited 30 min and 3 h after hypoxanthine administration, and in cerebral cortex this inhibitory effect was only observed at 24 h and 7 days after drug infusion. On the other hand, since antioxidant enzymes can also respond to sustained oxidative stress by a compensatory increase in their activities due to alteration of gene transcription (Travacio and Llesuy, 1996), the increase in catalase activity (18%) in striatum 7 days after hypoxanthine infusion observed in our study could be a consequence of enzymatic adaptation to enhanced free radical formation. Additional studies are necessary to elucidate such mechanisms, since this metabolic adaptation did not occur in the hippocampus and cerebral cortex.

Although extrapolation of findings from animal experiments to the human is difficult, it is conceivable that the differential role of hypoxanthine on these important enzyme activities in various brain structures might be associated with the neurological dysfunction present in patients affected by Lesch Nyhan disease.

Acknowledgements

This work was supported in part by grants from CNPq – Brazil, FAPERGS, RS-Brazil, and Programa de Núcleos de Excelência-Financiadora de Estudos e Projetos (PRONEX -Brazil).

References

1. Aebi, H., 1984. Catalase in vitro. Methods Enzymol. 105, 121-126.
2. Bavaresco, C.S., Zugno, A.I., Tagliari, B., Wannmacher, C.M.D., Wajner, M., Wyse A.T.S., 2004. Inhibition of Na^+, K^+ - ATPase activity in rat striatum by metabolites accumulated in Lesch Nyhan disease. Int. J. Devl. Neurosci. 22, 11-17.
3. Bavaresco, C.S., Chiarani, F., Matté, C., Wajner, M., Netto, C.A., Wyse, A.T.S., 2005. Effect of hypoxanthine on Na^+,K^+-ATPase activity and some parameters of oxidative stress in rat striatum. Brain Res. 1041, 198 – 204.
4. Bavaresco, C.S., Chiarani, F., Wannmacher, C.M.D., Netto, C.A., Wyse, A.T.S., 2006. Intrastriatal hypoxanthine reduces Na^+,K^+ - ATPase activity and induces oxidative stress in the rats. Metab. Brain Dis., in press.
5. Beal, M.F., 1995. Aging, energy, and oxidative stress in neurodegenerative diseases. Ann. Neurol. 38(3), 357-366.
6. Beckman, J.S., Liu, T.H., Hogan, E.L., Freeman, B.A., Hsu, C.Y., 1987. Oxygen free radicals and xanthine oxidase in cerebral ischemic injury in the rat. Soc. Neurosci. Abstr. 13, 1498.
7. Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein –die binding. Anal. Biochem. 72, 248 – 254.
8. Chan, K.M., Delfert, D., Junger, K.D., 1986. A direct colorimetric assay for Ca^+ -stimulated ATPase activity. Anal Biochem 157, 375 – 380.
9. Dasheiff, R.M., 1980. Benzodiazepinic treatment for Lesch-Nyhan syndrome? Dev. Med. Child. Neurol. 22, 101 – 102.

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

11. Erecinska, M., Silver, I.A., 1994. Ions and energy in mammalian brain. Prog. Neurobiol. 7, 21 – 29.

12. Floyd, R.A., 1999. Antioxidants, oxidative stress and degenerative neurological disorders. Proc. Soc. Exp. Biol. Med. 222, 236 – 245.

13. Garcia-Alloza, M., Gil-Bea, F.J., Diez-Ariza, M., Chen, C.P., Francis, P.T., Lasheras, B., Ramirez, M.J., 2005. Cholinergic-serotonergic imbalance contributes to cognitive and behavioral symptoms in Alzheimer's disease. Neuropsychologia 43, 442-449.

14. Grafius, M.A., Bond, H.E., Millar, D.B., 1971. Acetylcholinesterase interaction with a lipoprotein matrix. Eur. J. Biochem. 22, 382-390.

15. Grisar, T., 1984. Glial and neuronal Na^+,K^+-pump in epilepsy. Ann. Neurol. 16: 128 – 134.

16. Halliwell, B., 1996. Free radicals, proteins and DNA: oxidative damage versus redox regulation. Biochem. Soc. Trans. 24, 1023 – 1027.

17. Halliwell, B., Gutteridge, J.M., 1985. The importance of free radicals and catalytic metal ions in human diseases. Mol. Aspects Med. 8, 89-193.

18. Harkness, R.A., McCreanor, G.M., Watts, R.W., 1988. Lesch-Nyhan syndrome and its pathogenesis: purine concentrations in plasma and in urine with metabolite profiles in CSF. J. Inher. Metab. Dis. 11**,** 239 – 252.

19. Heimer, L., Zahm, D.S., Alheid, G.F., 1995. Basal Ganglia. In: Paxinos, G. (Ed), The Rat Nervous System. Academic Press, London, pp; 579-628.

20. Henderson, J.F., 1968. Possible functions of hypoxanthine-guanine phosphoribosyltransferase and their relation to the biochemical pathology of the Lesch-Nyhan syndrome. Fed. Proc. 27, 1075-1077.

21. Henderson, V.W., Watt, L., Buckwalter, J.G., 1996. Cognitive skills associated with estrogen replacement in women with Alzheimer's disease. Psychoneuroendocrinology 21, 421-430.

22. Hieber, V., Siegel, G.J., Fink, D.J., Beaty, M.W., Mata, M., 1991. Differential distribution of (Na, K)-ATPase alpha isoforms in the central nervous system. Cell. Mol. Neurobiol. 11, 253-262.

23. Horton, J.W., 2003. Free radicals and lipid peroxidation mediated injury in burn trauma: the role of antioxidant therapy. Toxicology 189, 75-88.

24. Jamme, I., Petit, E., Divoux, D., Gerbi, A., Maixent, J.M., Nouvelot, A., 1995. Modulation of mouse cerebral Na^{+},$K(^{+})$-ATPase activity by oxygen free radicals. Neuroreport 7, 333-337.

25. Jinnah, H.A., Friedmann, T., 2001. Lesch Nyhan disease and it variants. In: Scriver, C.R., Beaudet, A.L., Sly, W.S., Valle, D. (Eds), The Metabolic and Molecular Bases of Inherited Disease. McGraw-Hill, New York, pp. 2537-2569.

26. Jinnah, H.A., Gage, F.H., 1990. Animal models of Lesch-Nyhan syndrome. Brain Res. Bull. 25, 467 – 475.

27. Jones, D.H., Matus, A.I., 1974. Isolation of synaptic plasma membrane from brain by combined flotation-sedimentation density gradient centrifugation. Biochim. Biophys. Acta 356, 276 – 287.

28. Kisch, S.J., Fox, I.H., Kapur, B.M., Lloyd, K.G., Hornykiewicz, O., 1985. Brain benzodiazepine receptor binding and purine concentration in Lesch-Nyhan. Brain Res. 336, 117-123.

29. Lassiter, T.L., Barone, S.Jr., Padilla, S., 1998. Ontogenetic differences in the regional and cellular acetylcholinesterase and butyrylcholinesterase activity in the rat brain. Brain Res. Dev. Brain Res. 105, 109-123.

30. Lees, G.J., 1993. Contributory mechanism in the causation of neurodegenerative disorders. Neurosci. 54, 287 – 322.

31. Lloyd, K.G., Hornykiewicz, O., Davidson, L., Shannak, K., Farley, I., Goldstein, M., Shibuya, M., Kelley, W.N., Fox, I.H., 1981. Biochemical evidence of dysfunction of brain neurotransmitters in the Lesch-Nyhan syndrome. N. Engl. J. Med. 305, 1106-1111.

32. Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265 – 267.

33. Ma, M.H.Y., Stacey, N.C., Connolly, G.P., 2001. Hypoxanthine impairs morphogenesis and enhances proliferation of a neuroblastoma model of Lesch Nyhan syndrome. J. Neurosci. Res. 63, 500 – 508

34. Matthews, W.S., Solan, A., Barabas, G., Robey, K., 1999. Cognitive functioning in Lesch-Nyhan syndrome: a 4-year follow-up study. Dev. Med. Child. Neurol. 41, 260-262.

35. Mizuno, T., 1986. Long-term follow-up of ten patients with Lesch-Nyhan syndrome. Neuropediatrics 17, 158-161,

36. Nyhan, W.L., Oliver, W.J., Lesch, M., 1965. A familial disorder or uric acid metabolism and central nervous system function II. J. Pediatr. 67, 439 -444.

37. Oliveira, P.J., Rolo, A.P., Palmeira, C.M., Moreno, A.J., 2001. Carvedilol reduces mitochondrial damage induced by hypoxanthine/xanthine oxidase: relevance to hypoxia/reoxygenation injury. Cardiovasc. Toxicol. 1, 205-213.

38. Paxinos, G., Watson, C., 1986. The rat brain in stereotaxic coordinates. Academic Press, London.

39. Puig, J.G., Jimenez, M.L., Mateos, F.A., Fox, I.H., 1989. Adenine nucleotide turnover in hypoxanthine-guanine phosphoribosyl-transferase: evidence for an increased contribution of purine biosyntheses de novo. Metabolism 38, 410 – 418.

40. Puig, J.G., Mateos, F.A., 1993. The biochemical basis of HGPRT deficiency. In: Gresser, U. (Ed), Molecular genetics, biochemistry and clinical aspects of inherited disorders of purine and pyrimidine metabolism. Springer-Verlag, New York, pp. 12-26.

41. Rijksen, G., Staal, G.E.J., Van der Vlist, M.J.M., Beerner, F.A., Troost, J., Gutensohn, W., Van Laarhoven, J.P.R.M., De Bruyn, C.H.M.M., 1981. Partial hypoxanthine-guanine phosphoribosyl transferase deficiency with full expression of the Lesch – Nyhan syndrome. Hum. Gen. 57, 39 – 47.

42. Rosenbloom, R.M., Henderson, J.F., Caldwell, I.C., Kelley, W.N., Seegmiller, J.E., 1967. Inherited disorder of purine metabolism. JAMA 202, 175-177.

43. Saito, Y., Takashima, S., 2000. Neurotransmitter changes in the pathophysiology of Lesch-Nyhan syndrome. Brain Dev. 22, S122-131.

44. Stark, G., 2005. Functional consequences of oxidative membrane damage. J. Membr. Biol. 205, 1-16.

45. Travacio, M., Llesuy, S., 1996. Antioxidant enzymes and their modification under oxidative stress conditions. Free Radic. Res. Latin Am. 48, 9–13.

46. Villescas, R., Ostwald, R., Morimoto, H., Bennett, E.L., 1981. Effects of neonatal undernutrition and cold stress on behavior and biochemical brain parameters in rats. J. Nutr. 111, 1103-1110.

47. Visser, J.E., Bär, P.R., Jinnah, H.A., 2000. Lesch-Nyhan disease and the basal ganglia. Brain Res. Bull. 32, 449 – 475.

48. Visser, J.E., Smith, D.W., Moy, S.S., Breese, G.R., Friedmann, T., Rothstein, J.D., Jinnah, H.A., 2002. Oxidative stress and dopamine deficiency in a genetic mouse model of Lesch-Nyhan disease. Brain Res. Dev. Brain Res. 133, 127-139.

49. Wyse, A.T.S., Streck, E.L., Worm, P., Wajner, M., Ritter, F., Netto, C.A., 2000. Preconditioning prevents the inhibition of Na^+,K^+-ATPase activity after brain ischemia. Neurochem. Res. 25**,** 969 – 973.

Figure 1. Effect of intrastriatal hypoxanthine injection on Na^+, K^+ - ATPase activity in synaptic plasma membrane from striatum (A), hippocampus (B) and cerebral cortex (C) of rats at different periods after infusion. Data are means ± SD for four independent experiments (animals) performed in duplicate. ** $p<0.01$ compared to sham group (Student's *t* test). Hpx - hypoxanthine

Figure 2. Effect of intrastriatal hypoxanthine injection on acetylcholinesterase activity in striatum (A), cerebral cortex (B) and hippocampus (C) of rats at different periods after infusion. Data are means ± SD for four independent experiments (animals) performed in duplicate. **$p<0.01$; * $p<0.05$ compared sham group (Student's *t* test). Hpx – hypoxanthine.

Figure 3. Effect of intrastriatal hypoxanthine injection on catalase activity in striatum (A), cerebral cortex (B) and hippocampus (C) of rats at different periods after infusion. Data are means ± SD for 4-6 independent experiments (animals) performed in duplicate. ** $p<0.01$; * $p<0.05$ compared to sham group (Student's *t* test). Hpx – hypoxanthine.

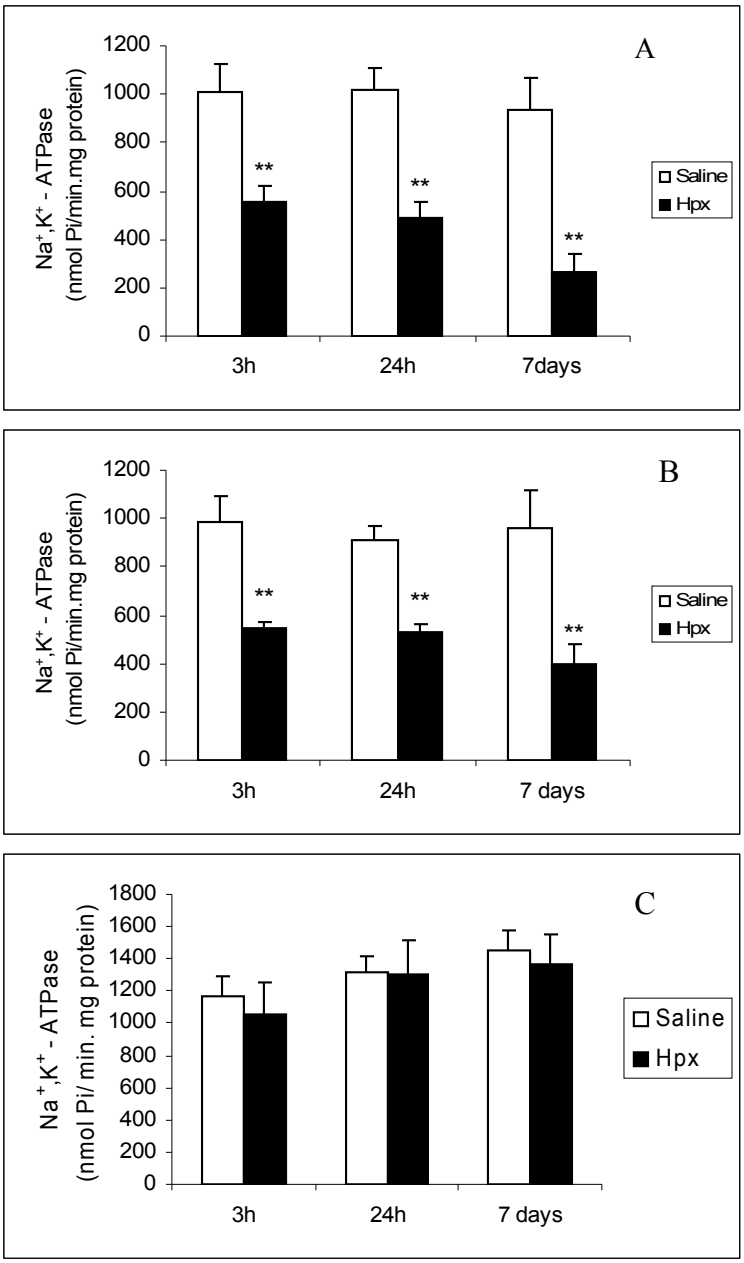
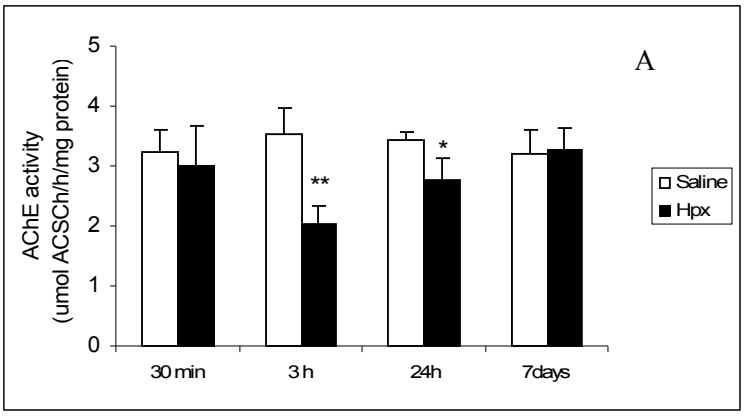
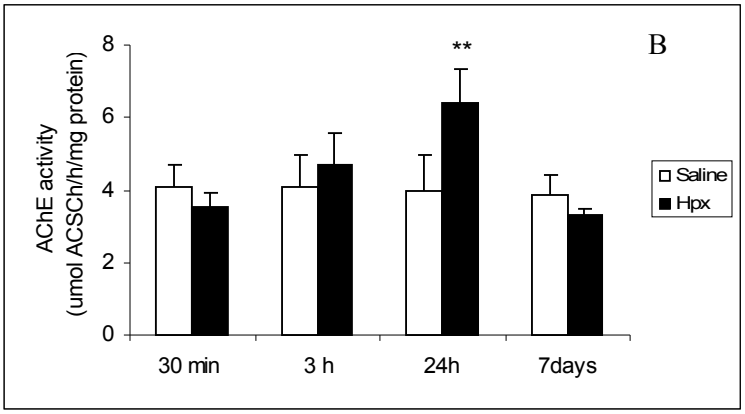

A
1200
1000
800
600
400
200
0
Na+,K+ - ATPase
(nmol Pi/min.mg protein)
**
**
**
3h
24h
7days
Saline
Hpx
B
1200
1000
800
600
400
200
0
Na+,K+ - ATPase
(nmol Pi/min.mg protein)
**
**
**
3h
24h
7 days
Saline
Hpx
C
1800
1600
1400
1200
1000
800
600
400
200
0
Na+,K+ - ATPase
(nmol Pi/ min. mg protein)
3h
24h
7 days
Saline
Hpx

Figure 1


A
AChE activity
(umol ACSCh/h/mg protein)
5
4
3
2
1
0
**
*
30 min
3 h
24h
7days
Saline
Hpx


B
AChE activity
(umol ACSCh/h/mg protein)
8
6
4
2
0
**
30 min
3 h
24h
7days
Saline
Hpx

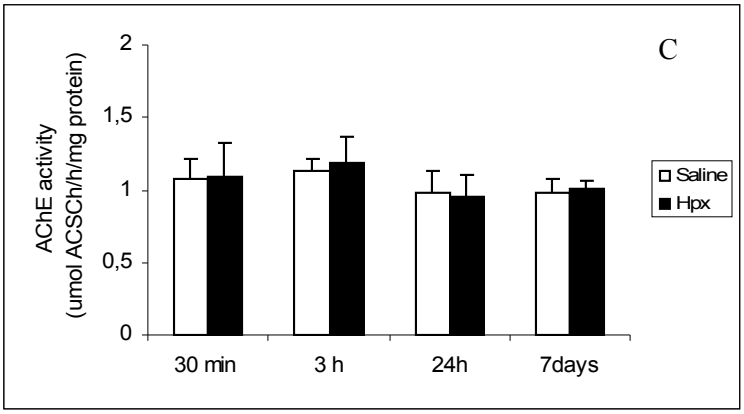
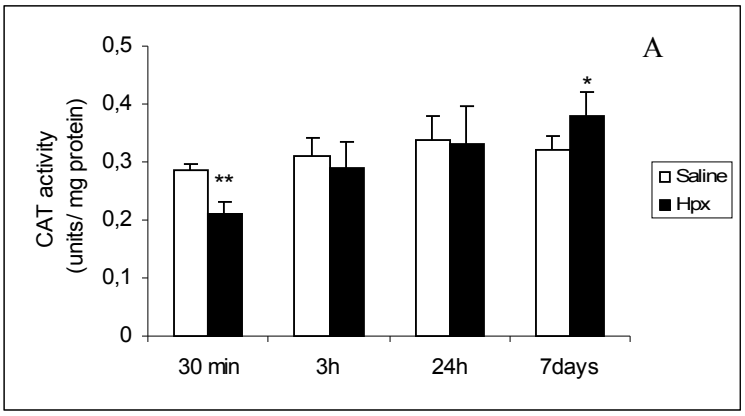
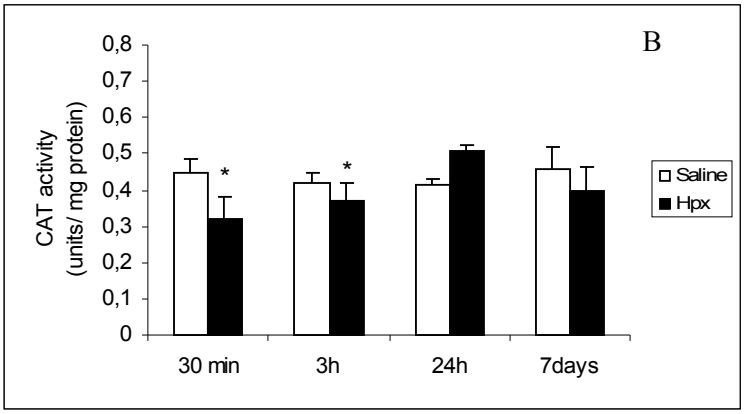

C
AChE activity
(umol ACSCh/h/mg protein)
2
1,5
1
0,5
0
30 min
3 h
24h
7days
Saline
Hpx

Figure 2


A
CAT activity
(units/ mg protein)
0,5
0,4
0,3
0,2
0,1
0
**
*
30 min
3h
24h
7days
Saline
Hpx


B
CAT activity
(units/ mg protein)
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
*
*
30 min
3h
24h
7days
Saline
Hpx

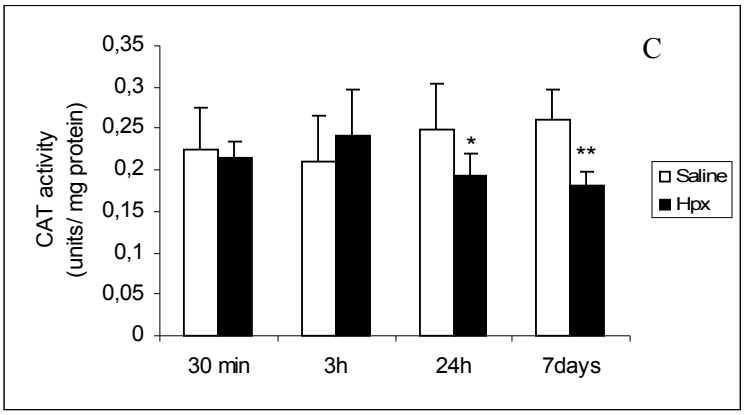

C
CAT activity
(units/ mg protein)
0,35
0,3
0,25
0,2
0,15
0,1
0,05
0
*
**
30 min
3h
24h
7days
Saline
Hpx

Figure 3

Capítulo III – Artigo 03

Biochemical effects of pretreatment with vitamins E and C in rats submitted to intrastriatal hypoxanthine administration

Caren Serra Bavaresco, Fabria Chiarani, Janaína Kolling,Carlos Alexandre Netto and Angela Terezinha de Souza Wyse

Aceito para publicação na revista Neurochemistry International , 2008

Biochemical effects of pretreatment with vitamins E and C in rats submitted to intrastriatal hypoxanthine administration

Caren Serra Bavaresco, Fabria Chiarani, Janaína Kolling, Carlos Alexandre Netto and Angela Terezinha de Souza Wyse[1]

Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil

[1]Address reprint request to: Dr. Angela T.S. Wyse, Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2600-Anexo, CEP 90035-003 Porto Alegre, RS, Brazil, Telephone number: 55 51 33085573; E-mail: wyse@ufrgs.br

Abstract

We previously demonstrated that intrastriatal injection of hypoxanthine, the major metabolite accumulating in Lesch Nyhan disease, inhibited Na^+,K^+-ATPase activity and induced oxidative stress in rat striatum. In the present study, we evaluated the action of vitamins E and C on the biochemical alteration induced by hypoxanthine administration on Na^+,K^+-ATPase, TBARS, TRAP, as well as on superoxide dismutase (SOD), catalase (CAT) and glutathione-peroxidase (GPx) activities in striatum of adult rats. Animals received pretreatment with vitamins E and C or saline during 7 days. Twelve hours after the last injection of vitamins or saline, animals were divided into two groups: (1) vehicle-injected group and (2) hypoxanthine-injected group. For all parameters investigated in this research, animals were sacrificed 30 min after drug infusion. Results showed that pretreatment with vitamins E and C prevented hypoxanthine-mediated effects on Na^+,K^+-ATPase, TBARS and antioxidant enzymes (SOD, CAT, GPx) activities; however the reduction on TRAP was not prevented by these vitamins. Although extrapolation of findings from animal experiments to humans is difficult, it is conceivable that these vitamins might serve as an adjuvant therapy in order to avoid progression of striatal damage in patients affected by Lesch Nyhan disease.

Key Words: Lesch-Nyhan; hypoxanthine; Na^+,K^+-ATPase; lipoperoxidation; vitamins E and C; antioxidants defenses.

1. Introduction

Severe alteration in purine salvage route due to deficiency of hypoxanthine-guanine phosphoribosyltranspherase activity causes Lesch Nyhan disease, leading to hypoxanthine accumulation in patients urine plasma and cerebrospinal fluid (Nyhan et al., 1965; Rosenbloom et al., 1967; Herderson et al., 1968; Rijksen et al., 1981; Harkness et al., 1988; Puig and Mateos, 1993; Jinnah and Friedmann, 2001). Hyperuricemia, cognitive deficits, striatal dopamine loss and self-mutilation behavior are the major signals and symptoms highlighted in this disease (Mizuno, 1986; Jinnah and Gage, 1990; Matthews et al., 1999; Jinnah and Friedmann, 2001). Although the underlying mechanisms of brain dysfunction in Lesch-Nyhan are poorly understood, tissue accumulation of oxypurines such as hypoxanthine has been proposed to contribute to the neurological dysfunction present in this disease (Dasheiff, 1980; Kisch et al., 1985; Ma et al., 2001; Visser et al., 2002).

Na^+, K^+ - ATPase (EC 3.6.1.37) is an enzyme embedded in the cell membrane, responsible for the generation of the membrane potential through the active transport of sodium and potassium ions in the central nervous system (CNS) necessary to maintain neuronal excitability. It is present at high concentrations in brain and consumes 40 – 50% of the ATP generated in this tissue (Erecinska and Silver, 1994). It has been demonstrated that the activity of this enzyme is inhibited in brain ischemia (Wyse at al., 2000), epilepsy (Grisar, 1984) and many neurodegenerative disorders (Lees, 1993**;** Kourie, 2001). Additionally, Na^+, K^+ - ATPase is inhibited by lipid peroxidation, free radicals and/or oxidative stress (Jamme et al., 1995; Rauchová et al., 1999; Zhang et al., 2007). On the other hand, a decrease on Na^+, K^+ ATPase activity in rat striatum 30 min

after hypoxanthine infusion was already demonstrated by our group (Bavaresco et al., 2007a). Moreover, we have also showed that hypoxanthine *in vitro* inhibited Na^+, K^+-ATPase activity in striatum of rats with 6 days of age (Bavaresco *et al*., 2004) and this effect seems to be associated with oxidative stress (Bavaresco *et al*., 2005).

Oxidative stress is defined as an imbalance between formation and removal of free radicals and it is presumed to be involved in many neurodegenerative events and in neurodegenerative diseases (Halliwell and Gutteridge, 1985; Beal, 1995; Dröge, 2002; Zhang et al., 2007). In this scenario, it has been demonstrated that hypoxanthine/xanthine oxidase acts as a source of oxidative stress in the vascular system (Oliveira et al, 2001) and might contribute to the destruction of blood-brain barrier observed in ischemic brain tissue (Beckman et al., 1987). In this perspective, evidence show that hypoxanthine administration also reduced total radical-trapping antioxidant parameter (TRAP) and increased chemiluminescence in hippocampus, cerebral cortex and striatum of rats (Bavaresco et al., 2007a).

It has been known that in order to defend themselves against oxidative damage, cells developed antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT) and glutathione-peroxidase (GPx). Cells also utilize non-enzymatic antioxidants defenses like vitamin E (α-tocopherol), vitamin C (ascorbic acid) and gluthatione (GSH) (Michiels et al., 1994; Halliwell, 2006). In this context, Bavaresco and colleagues (2006) show that CAT activity, an important antioxidant enzyme, was inhibited 30 min after intrastriatal hypoxanthine infusion in rat striatum. Moreover, a previous study from our group demonstrated that striatal homogenate preincubation with glutathione (GSH) and trolox (water-soluble vitamin E) were able to prevent Na^+, K^+-ATPase activity inhibition induced by hypoxanthine *in vitro* (Bavaresco et al., 2005).

Considering that (a) previous studies shows that intrastriatal hypoxanthine infusion decreases Na^+,K^+-ATPase, TRAP and catalase activities and increase chemiluminescence in rat striatum, (b) antioxidants prevent Na^+,K^+-ATPase activity inhibition elicited by hypoxanthine *in vitro;* we decided to investigate the influence of pretreatment with vitamins E and C on the effects elicited by intrastriatal administration of hypoxanthine on Na^+,K^+-ATPase, TBARS, TRAP, as well as on the antioxidant enzymes (CAT, SOD and GPx) activities. The drug was infused in striatum because patients with this syndrome present characteristic alterations in the basal ganglia (Jinnah and Friedmann, 2001).

2. Experimental Procedures

2.1. Animals and reagents

Seventy Wistar rats with 60 days of age (180-200g) were obtained from the Central Animal House of the Department of Biochemistry, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil. Animals were maintained on 12 h light/dark cycle (lights on from 7 a.m. to 7 p.m.) in an air-conditioned constant temperature (22^o C) colony room and had free access to a 20% (w/w) protein commercial chow and water. The "Principles of Laboratory Animal Care" (NIH publication 85-23, revised 1985) were followed for all the experiments and the protocols used were approved by the Ethics Committee for Animal Research of the Federal University of Rio Grande do Sul, Porto Alegre, Brazil.All chemicals were purchased from Sigma Chemical Co., St Louis, MO, USA.

2.2. Pretreatment with vitamins E and C

Rats were pretreated with daily intraperitonial (i.p) injections of α-tocopherol (40 mg/kg) and ascorbic acid (100 mg/kg), during 7 days. Controls animals received saline. α-Tocopherol and ascorbic acid doses and route of administration were chosen according to the protocols previously described by us and other investigators (Fighera et al., 1999; Wyse et al., 2002; Delwing et al., 2006).

2.3. Stereotaxic Surgery and placement of cannula

On the fifth day after pretreatment beginning, rats were anesthetized with ketamine and xilazine (75 and 10 mg/kg i.p., respectively) and placed in a rodent stereotaxic apparatus. Under stereotaxic guidance, a 27-gauge staineless cannula (0.9 mm O.D.) with an inner needle guide was inserted unilaterally into the right striatum (coordinates relative from bregma: AP, -0.5 mm; ML -2.5 mm; V -2.5 mm from the dura) (Paxinos and Watson, 1986). The cannulae were fixed to the skull with dental cement. Two days after surgery, a 30–gauge needle was inserted into the guide cannula in order to inject 2 μL of buffered hypoxanthine (10 μM) or vehicle (saline) into the right striatum over a 1 min interval. Rats were divided into four groups as follows: group 1, rats that received intrastriatal saline and i.p. saline solution; group 2, rats that received intrastriatal hypoxanthine solution (20 pmol/ 2μl) and i.p. saline solution; group 3, rats that received intrastriatal saline and i.p. vitamins E and C solution and group 4, rats that received intrastriatal hypoxanthine solution and i.p. vitamins E and C solution. Animals were sacrificed 30 min after drug administration. Hypoxanthine dose was chosen according to Puig and colleagues (1989). The drug was infused into the striatum because patients with this disease present characteristic alterations in the basal ganglia (Jinnah and Friedmann, 2001).

2.4. Tissue preparation

Animals were killed by decapitation without anesthesia, the brain was removed and striatum was dissected out. For Na^+, K^+-ATPase activity determination, striatum were homogenized in 10 volumes (w/v) of 0.32 M sucrose solution containing 5.0 mM HEPES (pH 7.45) and 1.0 mM EDTA, pH 7.4 (Wyse *et al*., 2000). For CAT activity assay, striatum was homogenized in 10 volumes (w/v) of 10.0 mM potassium phosphate buffer, pH 7.6 (Aebi, 1984). For SOD activity assay, striatum was homogenized in 10 volumes (w/v) of 50.0 mM Tris-HCl buffer with 1.00 mM EDTA, pH 8.2 (Marklund, 1985). For GPx activity assay, striatum was homogenized in 10 volumes (w/v) of 100.0 mM potassium phosphate buffer with 1.00 mM EDTA, pH 7.7 (Wendel, 1981). For TRAP determinations, the same cerebral structure was homogenized in 10 volumes (1:10) of 0.1 M glycine buffer, pH 8.6 (Evelson et al., 2001). For TBARS assay, striatum was homogenized in 10 volumes (w/v) of 1.15% KCl, pH 7.6 (Ohkawa et al., 1979).

2.5. Preparation of synaptic plasma membrane from striatum.

Synaptic plasma membranes were prepared according to the method of Jones and Matus (1974), with some modifications (Wyse *et al*., 2000). Striatum homogenate was centrifuged at 1,000 g for 20 min and the supernatant removed and centrifuged at 12,000 x g for a further 20 min. The pellet was then resuspended in hypotonic buffer (5.0 mM Tris-HCl buffer, pH 8.1), incubated at 0^0C for 30 min and applied on a discontinuous sucrose density gradient consisting of successive layers of 0.3, 0.8 and 1.0 M. After centrifugation at 69,000 x *g* for 2 h, the fraction at the 0.8–1.0 M sucrose interface was taken as the membrane enzyme preparation.

2.6. *Determination of Na^+, K^+ - ATPase activity*

The reaction mixture for the Na^+,K^+-ATPase assay contained 5.0 mM $MgCl_2$, 80.0 mM NaCl, 20.0 mM KCl, and 40.0 mM Tris-HCl buffer, pH 7.4, in a final volume of 200 µL. The reaction was started by the addition of ATP (disodium salt, vanadium free) to a final concentration of 3.0 mM. Controls were assayed under the same conditions with the addition of 1.0 mM ouabain. Na^+,K^+-ATPase activity was calculated by the difference between the two assays as described by Wyse and colleagues (2000). Released inorganic phosphate (Pi) was measured by the method of Chan and colleagues (1986); enzyme specific activity was expressed as nmol Pi released per min per mg of protein.

2.7. TBARS assay

TBARS was determined according to the method described by Ohkawa et al. (1979). Briefly, 50 µl of 8.1% sodium dodecyl sulfate, 1.5 mL of 20% acetic acid solution adjusted to pH 3.5 and 1.5 mL of 0.8% aqueous solution of thiobarbituric acid were added to 500 µl of striatum homogenate in a Pyrex tube, and then heated in a boiling water bath for 60 min. After cooling with tap water, the mixture was centrifuged at 1000 X g for 10 min. The organic layer was taken and the resulting pink color was determined in a spectrophotometer at 535 nm. The results were reported as nmol TBARS/mg protein.

2.8. *TRAP assay*

TRAP assay measures the total antioxidant capacity of the tissue and was determined by measuring the luminol chemiluninescence intensity induced by 2,2´-Azo-

bis (2-amidinopropane) (ABAP), according to the method of Evelson and colleagues (2001). Briefly, 4.0 ml of 10.0 mM ABAP were added to the vial and background chemiluminescence was measured. Ten µl of 0.2 µM trolox or homogenates (1:10 in 0.1 M glycine buffer, pH 8.6) were added and chemiluminescence was measured until it reached the initial levels. The addition of trolox or tissue homogenate to the incubation medium reduces the chemiluminescence. The time necessary for return to the levels present before the addition was considered to be the induction time (IT). IT is directly proportional to the antioxidant capacity of the tissue and was compared to IT of trolox. Results were represented as nM trolox / mg protein.

2.9. SOD activity assay

This method for the assay of SOD activity is based on the capacity of pyrogallol to autoxidize, a process highly dependent on O^{2-}, which is substrate for SOD (Marklund, 1985). The inhibition of autoxidation of this compound occurs in the presence of SOD, whose activity can be then indirectly assayed spectrophotometrically at 420 nm, using a double-beam spectrophotometer with temperature control (Hitachi U-2001). A calibration curve was performed with purified SOD as standard, in order to calculate the activity of SOD present in the samples. The results were reported as units of SOD/mg protein.

2.10. CAT activity assay

CAT activity was assayed by the method of Aebi (1984), which is based on the disappearance of H_2O_2 at 240 nm. One unit of the enzyme is defined as 1 µmol of hydrogen peroxide consumed per minute and the specific activity was reported as units per mg protein.

2.11. GPx activity assay

GPx activity was measured using tert-butyl-hydroperoxide as substrate (Wendel, 1981). NADPH disappearance was monitored at 340 nm using a double-beam spectrophotometer with temperature control (Hitachi U-2001). The medium contained 2 mM glutathione, 0.15 U/mL glutathione reductase, 0.4 mM azide, 0.5 mM tert-butyl-hydroperoxide and 0.1 mM NADPH. One GPx unit is defined as one μmol of NADPH consumed per minute and the specific activity is represented as units per mg protein.

2.12. Protein determination

Protein was measured by the method of Lowry et al. (1951) using bovine serum albumin as standard.

2.13. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) followed by the Duncan´s multiple range test when the F-test was significant. All analyses were performed using the Statistical Package for the Social Science (SPSS) software in a PC-compatible computer. A value of $p<0.05$ was considered to be significant.

3. Results

3.1. Experiment 1: Effect of vitamins E and C on Na^+,K^+-ATPase activity 30 min after intrastriatal hypoxanthine infusion:

Figure 1 shows the effect of pretreatment with vitamins E and C on Na^+, K^+ -ATPase activity in rat striatum after intrastriatal injection of hypoxanthine. Na^+, K^+ -

ATPase activity was significantly reduced in the ipsislateral striatum (53%) 30 min after hypoxanthine administration. Vitamins E and C *per se* did not alter enzyme activity; however supplementation with vitamins E and C totally prevented Na^+, K^+ - ATPase activity inhibition mediated by hypoxanthine [$F(3,12)=13.748$; $p<0.05$].

3.2. Experiment 2: Effect of vitamins E and C administration on TBARS and TRAP in rat striatum after intrastriatal hypoxanthine infusion:

Since we have already demonstrated that hypoxanthine induces oxidative stress in brain of rats (Bavaresco et al., 2007a), we also verified the effect of vitamins E and C on TBARS, an index of lipoperoxidation, and on TRAP, a parameter of tissue antioxidant capacity. Figure 2A shows an increase on TBARS index (32%) 30 min after hypoxanthine administration in ipsislateral striatum. *Post hoc* analysis shows that vitamin E and C *per se* did not alter TBARS, but prevented the increase of TBARS caused by hypoxanthine [$F(3,16)=4.061$; $p<0.05$]. Concerning about tissue antioxidant capacity, TRAP was significantly reduced (46%) in rat ipsislateral striatum 30 min after hypoxanthine infusion. Interestingly, vitamins E and C were no able to prevent TRAP decrease in our experimental conditions [$F(3,16)=10.204$; $p<0.05$] (Figure 2B).

3.3. Experiment 3: Effect of vitamins E and C and the intrastriatal hypoxanthine infusion on SOD, CAT and GPx activities:

Next, we evaluated the role of vitamins E and C on the effects elicited by intrastriatal hypoxanthine on SOD, CAT and GPx activities. Figure 3A shows that SOD activity was significantly reduced (31%) in the ipsislateral striatum however supplementation with vitamins E and C totally prevented this enzyme inhibition [$F(3,12)=3.276$; $p<0.05$]. Figure 3B shows that 30 min after hypoxanthine infusion CAT

activity was reduced (43%) in striatum of rats. *Post hoc* analysis shows that vitamin E and C *per se* did not alter CAT activity, but totally prevented the effect inhibitory effect elicited by hypoxanthine on CAT activity [$F(3,12)=7.025$; $p<0.05$]. In contrast, Figure 3C shows that GPx activity was increased (33%) after oxypurine infusion in the same cerebral structure. Vitamin E and C *per se* did not alter GPx activity, but prevented the effect of hypoxanthine on GPx activity [$F(3,16)=4.061$; $p<0.05$]

4. Discussion

Lesch Nyhan is an X-linked recessive disease of purine metabolism (Nyhan et al., 1965; Jinnah and Friedmann, 2001). It is biochemically characterized by deficiency of HPRT activity, resulting in tissue accumulation of hypoxanthine. Patients present neurological and cognitive disabilities, choreoatetosis, spasticity, mental retardation and characteristic neurobehavioral phenotype characterized by compulsive self-mutilation (Jinnah and Friedmann, 2001). We have already reported that intrastriatal hypoxanthine administration inhibited Na^+,K^+-ATPase activity in striatum, cerebral cortex and hippocampus of rats and also elicits oxidative stress in rat brain (Bavaresco et al., 2007a). Moreover, it has been demonstrated an impairment on spatial memory in Water Maze task in rats treated with hypoxanthine (Bavaresco et al., 2007b), as well as that GSH and trolox *in vitro* were able to prevent Na^+,K^+-ATPase activity inhibition and also the TBARS increase caused by hypoxanthine in striatum of neonate rats (Bavaresco et al., 2005).

Oxidative stress seems to be involved in many neurodegenerative disorders (Halliwell, 1996; Zarkovic, 2003; Behl, 2005). It has been shown that brain is highly susceptible to oxidative stress because the elevated rate of oxygen consumption,

presence of high levels of polyunsaturated fatty acids and low cerebral antioxidant defenses compared to other tissues (Floyd, 1999; Halliwell, 2006), a fact that makes it more vulnerable to reactive oxygen species. In this perspective, strategies to prevent brain oxidative damage seem to be necessary. In this context, both water-soluble (vitamin C) and lipid soluble (vitamin E) nutrients comprise an important aspect on the antioxidant defense system, particularly to brain cells (Zaidi and Banu, 2004).

α-Tocopherol has been shown to promote protection to cells exposed to oxidative stress damage by scavenging free radicals, stabilizing membranes and blocking the cascade of biochemical routs involved on cellular necrosis (Kelly, 1998). α-Tocopherol is converted to tocopheryl radical, requiring ascorbate for its regeneration to reduced tocopherol (Frei et al., 1999). This combination of α-tocopherol and ascorbic acid because has proven to be effective in preventing biochemical and behavioral deficits produced in animal models of metabolic diseases, as well as in age-related motor and memory deficit of rats (Bickford et al., 2000; Stefanello et al., 2005). In this context, it has been demonstrated that supplementation with vitamins E and C prevented Na^+,K^+-ATPase activity inhibition (Wyse et al., 2002; Bavaresco et al. 2003; Monteiro et al., 2007; Zugno et al., 2007), and memory/learning impairment (Engelhart et al., 2002; Monteiro et al., 2005; Delwing et al., 2006; Wengreen et al., 2007) in humans and in animal models.

In the present study we firstly investigated the influence of vitamins E and C on the inhibitory effect provoked by intrastriatal injection of hypoxanthine on Na^+,K^+-ATPase activity in rat striatum. Results confirmed our previous studies showing that intrastriatal hypoxanthine injection reduces Na^+,K^+-ATPase activity (53%) in rat striatum. We also verified that vitamins E and C *per se* did not alter Na^+,K^+-ATPase activity, however prevented enzyme inhibition provoked by hypoxanthine

administration. It has been reported that decreased activity of Na^+,K^+-ATPase is a cause/consequence of peroxidative process (Matté, et al., 2006; Zhang et al., 2007). Such effects could be depend on decreased membrane fluidity, membrane lipidic composition modification and also oxidation of SH-groups essentials for Na^+,K^+-ATPase activity (Zhang et al., 2007). In this context, previous data indicated that, after 30 min of hypoxanthine administration, SH-groups modifications are not involved in inhibition of Na^+, K^+-ATPase activity since total thiol reduced content in rat striatum was not altered by hypoxanthine infusion (Bavaresco et al., 2007a). Moreover, since vitamin E interacts with cell membranes, interrupts chain oxidative reactions and protect polyunsaturated fatty acids oxidation, protective effects on Na^+, K^+-ATPase activity mediated by vitamins E and C could be related to inhibition of lipid peroxidation and/or free radical generation caused by hypoxanthine.

Considering that previous studies in a knockout mouse model of Lesch – Nyhan disease showed an induction of oxidative stress (Visser et al., 2002) and that *in vitro* and *in vivo* studies demonstrated that hypoxanthine increases TBARS (parameter of lipid peroxidation) and decreases TRAP (parameter of non-enzymatic antioxidant capacity) (Bavaresco et al., 2005, Bavaresco et al., 2007a), in the present study we also analyzed the role of vitamins E and C on preventing modifications mediated by hypoxanthine on some oxidative stress parameters namely, TBARS and TRAP. Hypoxanthine administration increased TBARS (32%) and reduced TRAP (46%). Results also indicated that vitamins *per se* were ineffective to alter any parameter tested; however pretreatment with vitamins E and C prevented the increase on TBARS, but did not prevent TRAP reduction.

Initially, it is important to analyze results in a global perspective. It has been shown that enhance in susceptibility to oxidative stress into tissue is correlated to the

decrease in antioxidant defenses (Evelson et al., 2001). Accordingly to TRAP technique, values obtained in this test reflect, principally, tissue GSH content. Under these circumstances, it is possible that we can't observed the protective role of vitamins E and C on TRAP in rat striatum since 50% of TRAP values obtained are related to GSH content (Evelson et al., 2001) and vitamins E and C could be acting in others antioxidants compounds rather than on GSH content. On the other hand, vitamins E and C were able to block hypoxanthine-induced increase on TBARS, an index of lipid peroxidation. This evidence suggests that vitamins E and C may reduce lipoperoxidation mediated by hypoxanthine on cellular membranes.

We also tested the influence of co-administration of vitamins E and C on SOD, CAT and GPX activities in rat striatum after hypoxanthine infusion. Hypoxanthine administration reduced SOD (31%) and CAT (43%) activities but enhanced GPx activity (33%) in rat striatum. Results showed that vitamins *per se* did not alter any parameter tested however pretreatment with vitamins E and C prevented the effects elicited by hypoxanthine on enzymes activities. Despite the fact that antioxidant enzymes are present in various amounts depending on cell type, it was possible that antioxidant enzymes influence each other to guarantee neuronal protection (Michiels et al., 1994; Travacio and Llesuy, 1996). Accordingly, SOD dismutates radical superoxide into hydrogen peroxide and seems to be the first line defense against oxidative stress (Michiels et al., 1994). In our study, results show that hypoxanthine inhibits SOD activity in rat striatum. So far, this could be associated to the fact that hypoxanthine is converted to xanthine and uric acid by xanthine oxidase, producing superoxide anion and oxygen-derived free radicals (Horton, 2003). In fact, peroxide and free radicals formed by hypoxanthine could mediate SOD activity inhibition observed in this research. Furthermore, it has been demonstrated that inhibition of SOD activity increase

superoxide levels which in the presence of hydrogen peroxide could undergo iron-catalyzed-Haber-Weiss reaction, giving rise to the highly reactive hydroxyl radical (Ito et al., 1996). In this situation, balance between CAT and GPx activities was determinative on keeping cellular integrity since both enzymes reduce hydrogen peroxide into O_2 and H_2O (Halliwell, 1996; Delwing et al., 2006). In this study, we verified an increase on GPx activity and a reduction on CAT activity after hypoxanthine administration. Although we can´t precisely postulate the mechanism that resulted on GPx activity increase, it is possible that a compensatory effect on enzymatic activity had occurred to overcome the inhibition on CAT activity elicited by hypoxanthine administration. Moreover, GPx also reduces lipid peroxides, thereby protecting hydrophobic membranes from oxidative stress (Michiels et al., 1994). Since hypoxanthine induced lipid peroxidation as indicated by the increase on TBARS assay (Bavaresco et al., 2006), it is possible to speculate that GPx activity increase observed in this study occurred trough a cellular strategy in order to protect biological membranes against oxidative lipid damage. On the other hand, none of this enzymatic modification could be visualized after vitamins E and C administration. In this perspective, vitamin E and C supplementation probably reduced hypoxanthine-elicited free radical formation in rat striatum.

In summary, it has been shown that pretreatment with vitamins E and C prevented the reduction on Na^+, K^+-ATPase activity and the induction of oxidative stress observed 30 min after intrastriatal hypoxanthine infusion. Although extrapolation of experimental findings to human conditions is difficult, it is conceivable that antioxidants may have a protective role against toxic effects of hypoxanthine and might serve as an adjuvant therapy in order to avoid progression of striatal damage in patients affected by Lesch Nyhan disease.

Acknowledgements

This work was supported in part by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq-Brazil) and by the FINEP Research Grant ‘‘Rede Instituto Brasileiro de Neurociência (IBN-Net) # 01.06.0842-00’’.

References

Aebi, H., 1984. Catalase in vitro. Methods Enzymology 105, 121-126.

Bavaresco, C.S., Calcagnotto, T., Tagliari, B., Delwing, D., Lamers, M.L., Wannmacher, C.M., Wajner, M., Wyse, A.T., 2003. Brain Na^+,$K(^+)$-ATPase inhibition induced by arginine administration is prevented by vitamins E and C. Neurochemistry Research 28, 825-829.

Bavaresco, C.S., Zugno, A.I., Tagliari, B., Wannmacher, C.M.D., Wajner, M., Wyse A.T.S., 2004. Inhibition of Na^+, K^+ - ATPase activity in rat striatum by metabolites accumulated in Lesch Nyhan disease. International Journal of Developmental Neuroscience 22, 11-17.

Bavaresco, C.S., Chiarani, F., Matté, C., Wajner, M., Netto, C.A., Wyse, A.T.S., 2005. Effect of hypoxanthine on Na^+,K^+-ATPase activity and some parameters of oxidative stress in rat striatum. Brain Research 1041, 198 – 204.

Bavaresco, C.S., Chiarani, F., Wajner, M., Netto, C.A., de Souza Wyse, A.T. 2006. Intrastriatal hypoxanthine administration affects Na^+,K^+-ATPase, acetylcholinesterase and catalase activities in striatum, hippocampus and cerebral cortex of rats. International Journal of Developmental Neuroscience 24, 411-417

Bavaresco, C.S., Chiarani, F., Wannmacher, C.M., Netto, C.A., Wyse, A.T. 2007a. Intrastriatal hypoxanthine reduces Na(+),K (+)-ATPase activity and induces oxidative stress in the rats. Metabolic Brain Disease 22, 1-11.

Bavaresco, C.S., Chiarani, F., Duringon, E., Ferro, M.M., Cunha, C.D., Netto, C.A., Wyse, A.T. 2007b. Intrastriatal injection of hypoxanthine reduces striatal serotonin content and impairs spatial memory performance in rats. Metabolic Brain Disease 22, 67-76.

Beal, M.F., 1995. Aging, energy, and oxidative stress in neurodegenerative diseases. Annals Neurology 38, 357-366.

Beckman, J.S., Liu, T.H., Hogan, E.L., Freeman, B.A., Hsu, C.Y., 1987. Oxygen free radicals and xanthine oxidase in cerebral ischemic injury in the rat. Society Neuroscience Abstract 13, 1498.

Behl, P., Stefurak, T.L., Black, S.E., 2005. Progress in clinical neurosciences: cognitive markers of progression in Alzheimer's disease. Canadian Journal Neurological Science 32, 40-151. Review.

Bickford, P.C., Gould, T., Briederick, L., Chadman, K., Pollock, A., Young, D., Shukitt-Hale, B., Joseph, J., 2000. Antioxidant-rich diets improve cerebellar physiology and motor learning in aged rats. Brain Research 866(1-2), 211-277.

Chan, K.M., Delfert, D., Junger, K.D., 1986. A direct colorimetric assay for Ca^{+} -stimulated ATPase activity. Analytical Biochemistry 157, 375 – 380.

Dasheiff, R.M., 1980. Benzodiazepinic treatment for Lesch-Nyhan syndrome? Developmental Medicine and Child Neurology 22, 101 – 102.

Delwing, D., Bavaresco, C.S., Monteiro, S.C., Matté, C., Netto, C.A., Wyse, A.T., 2006. Alpha-Tocopherol and ascorbic acid prevent memory deficits provoked by chronic hyperprolinemia in rats. Behavioral Brain Research 168, 185-189.

Dröge, W., 2002. Free radicals in the physiological control of cell function. Physiological Reviews. 8, 47-95. Review.

Engelhart, M.J., Geerlings, M.I., Ruitenberg, A., Van Swieten, J.C., Hofman, A., Witteman, J.C., Breteler, M.M., 2002. Diet and risk of dementia: Does fat matter?: The Rotterdam Study. Neurology 59, 1915-1921.

Erecinska, M., Silver, I.A., 1994. Ions and energy in mammalian brain. Progress in Neurobiology 7, 21 – 29.

Evelson, P., Travacio, M., Repetto, M., Escobar, J., Llesuy, S., Lissi, E.A., 2001. Evaluation of total reactive antioxidant potential (TRAP) of tissue homogenates and their cytosols. Archives of Biochemistry and Biophysics 388, 261-266.

Fighera, M.R., Queiroz, C.M., Stracke, M.P., Brauer, M.C., González-Rodríguez, L.L., Frussa-Filho, R., Wajner, M., de Mello, C.F., 1999. Ascorbic acid and alpha-tocopherol attenuate methylmalonic acid-induced convulsions. Neuroreport 10, 2039-2043.

Floyd, R.A., 1999. Antioxidants, oxidative stress and degenerative neurological disorders. Proceedings of the Society for Experimental Biology and Medicine 222, 236 – 245.

Frei, B., 1999. On the role of vitamin C and other antioxidants in atherogenesis and vascular dysfunction. Proceedings of the Society for Experimental Biology and Medicine 222, 196-204. Review.

Grisar, T., 1984. Glial and neuronal Na^+,K^+-pump in epilepsy. Annals Neurology 16, 128 – 134.

Halliwell, B., Gutteridge, J.M., 1985. The importance of free radicals and catalytic metal ions in human diseases. Molecular Aspects of Medicine 8, 89-193.

Halliwell, B., 1996. Free radicals, proteins and DNA: oxidative damage versus redox regulation. Biochemistry Society Transactions 24, 1023 – 1027.

Halliwell B., 2006. Oxidative stress and neurodegeneration: where are we now?. Journal of Neurochemistry 97, 1634-1658. Review.

Harkness, R.A., McCreanor, G.M., Watts, R.W., 1988. Lesch-Nyhan syndrome and its pathogenesis: purine concentrations in plasma and in urine with metabolite profiles in CSF. Journal of Inherited Metabolic Disease 11**,** 239 – 252.

Henderson, J.F., 1968. Possible functions of hypoxanthine-guanine phosphoribosyltransferase and their relation to the biochemical pathology of the Lesch-Nyhan syndrome. Federation Proceedings 27, 1075-1077.

Horton, J.W., 2003. Free radicals and lipid peroxidation mediated injury in burn trauma: the role of antioxidant therapy. Toxicology 189, 75-88.

Ito, Y., Pagano, P.J., Tornheim, K., Brecher, P., Cohen, R.A., 1996. Oxidative stress increases glyceraldehyde-3-phosphate dehydrogenase mRNA levels in isolated rabbit aorta. American Journal of Physiology. 270, 81-87.

Jamme, I., Petit, E., Divoux, D., Gerbi, A., Maixent, J.M., Nouvelot, A., 1995. Modulation of mouse cerebral Na^{+},$K(^{+})$-ATPase activity by oxygen free radicals. Neuroreport 7, 333-337.

Jinnah, H.A., Friedmann, T., 2001. Lesch Nyhan disease and it variants. In: Scriver, C.R., Beaudet, A.L., Sly, W.S., Valle, D. (Eds), The Metabolic and Molecular Bases of Inherited Disease. McGraw-Hill, New York, pp. 2537-2569.

Jinnah, H.A., Gage, F.H., 1990. Animal models of Lesch-Nyhan syndrome. Brain Research Bulletin 25, 467 – 475.

Jones, D.H., Matus, A.I., 1974. Isolation of synaptic plasma membrane from brain by combined flotation-sedimentation density gradient centrifugation. Biochimica Et Biophysica Acta 356, 276 – 287.

Kelly F.J., 1998. Use of antioxidants in the prevention and treatment of disease. Journal of the International Federation of Clinical Chemistry 10, 21-23. Review.

Kisch, S.J., Fox, I.H., Kapur, B.M., Lloyd, K.G., Hornykiewicz, O., 1985. Brain benzodiazepine receptor binding and purine concentration in Lesch-Nyhan. Brain Research 336, 117-123.

Kourie, J.I. 2001. Mechanisms of amyloid beta protein-induced modification in ion transport systems: implications for neurodegenerative diseases. Cellular and Molecular Neurobiology 21, 173-213.

Lees, G.J., 1993. Contributory mechanism in the causation of neurodegenerative disorders. Neuroscience 54, 287 – 322.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry 193, 265 – 267.

Marklund, S.L., 1985. Product of extracellular-superoxide dismutase catalysis. FEBS Letters 184, 237-239.

Matté, C., Durigon, E., Stefanello, F.M., Cipriani, F., Wajner, M., Wyse, A.T., 2006. Folic acid pretreatment prevents the reduction of Na(+),K(+)-ATPase and butyrylcholinesterase activities in rats subjected to acute hyperhomocysteinemia. International Journal of Developmental Neuroscience 24, 3-8.

Matthews, W.S., Solan, A., Barabas, G., Robey, K., 1999. Cognitive functioning in Lesch-Nyhan syndrome: a 4-year follow-up study. Developmental Medicine and Child Neurology 41, 260-262.

Michiels, C., Raes, M., Toussaint, O., Remacle, J., 1994. Importance of Se-glutathione peroxidase, catalase, and Cu/Zn-SOD for cell survival against oxidative stress. Free Radical Biology and Medicine 17, 235-248. Review

Mizuno, T., 1986. Long-term follow-up of ten patients with Lesch-Nyhan syndrome. Neuropediatrics 17, 158-161.

Monteiro, S.C., Matté, C., Bavaresco, C.S., Netto, C.A., Wyse, A.T., 2005. Vitamins E and C pretreatment prevents ovariectomy-induced memory deficits in water maze. Neurobiology of Learning and Memory 84, 192-199.

Monteiro, S.C., Mattos, C.B., Scherer, E.B., Wyse, A.T., 2007. Supplementation with vitamins E plus C or soy isoflavones in ovariectomized rats: effect on the activities of Na(+), K (+)-ATPase and cholinesterases. Metabolic Brain Disease 22, 156-171.

Nyhan, W.L., Oliver, W.J., Lesch, M., 1965. A familial disorder or uric acid metabolism and central nervous system function II. Journal of Pediatrics 67, 439 -444.

Ohkawa, H., Ohishi, N., Yagi, K., 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Analytical Biochemistry 95, 351-358.

Oliveira, P.J., Rolo, A.P., Palmeira, C.M., Moreno, A.J., 2001. Carvedilol reduces mitochondrial damage induced by hypoxanthine/xanthine oxidase: relevance to hypoxia/reoxygenation injury. Cardiovascular Toxicolology 1, 205-213.

Paxinos, G., Watson, C., 1986. The rat brain in stereotaxic coordinates. Academic Press, London.

Puig, J.G., Jimenez, M.L., Mateos, F.A., Fox, I.H., 1989. Adenine nucleotide turnover in hypoxanthine-guanine phosphoribosyl-transferase: evidence for an increased contribution of purine biosyntheses de novo. Metabolism 38, 410 – 418.

Puig, J.G., Mateos, F.A., 1993. The biochemical basis of HGPRT deficiency. In: Gresser, U. (Ed), Molecular genetics, biochemistry and clinical aspects of inherited

disorders of purine and pyrimidine metabolism. Springer-Verlag, New York, pp. 12-26.

Rauchová, H., Drahota, Z., Koudelová, J., 1999. The role of membrane fluidity changes and thiobarbituric acid-reactive substances production in the inhibition of cerebral cortex Na^+/K^+-ATPase activity. Physiological Research 48, 73-78.

Rijksen, G., Staal, G.E.J., Van der Vlist, M.J.M., Beerner, F.A., Troost, J., Gutensohn, W., Van Laarhoven, J.P.R.M., De Bruyn, C.H.M.M., 1981. Partial hypoxanthine-guanine phosphoribosyl transferase deficiency with full expression of the Lesch – Nyhan syndrome. Human Genetics 57, 39 – 47.

Rosenbloom, R.M., Henderson, J.F., Caldwell, I.C., Kelley, W.N., Seegmiller, J.E., 1967. Inherited disorder of purine metabolism. JAMA 202, 175-177.

Stefanello, F.M., Franzon, R., Tagliari, B., Wannmacher, C., Wajner, M., Wyse, A.T., 2005. Reduction of butyrylcholinesterase activity in rat serum subjected to hyperhomocysteinemia. Metabolic Brain Disease 20, 97-103.

Travacio, M., Llesuy, S., 1996. Antioxidant enzymes and their modification under oxidative stress conditions. Free Radical Research in Latin America 48, 9–13.

Vatassery G.T., 1998. Vitamin E and other endogenous antioxidants in the central nervous system. Geriatrics. 53, S25-27. Review.

Wengreen, H.J., Munger, R.G., Corcoran, C.D., Zandi, P., Hayden, K.M., Fotuhi, M., Skoog, I., Norton, M.C., Tschanz, J., Breitner, J.C., Welsh-Bohmer, K.A., 2007. Antioxidant intake and cognitive function of elderly men and women: the Cache County Study. The journal of nutrition, health and aging 11, 230-237.

Visser, J.E., Smith, D.W., Moy, S.S., Breese, G.R., Friedmann, T., Rothstein, J.D., Jinnah, H.A., 2002. Oxidative stress and dopamine deficiency in a genetic mouse

model of Lesch-Nyhan disease. Brain Research in Developmental Brain Research 133, 127- 139.

Wendel, A., 1981. Glutathione peroxidase. Methods Enzymology 77, 325-333.

Wyse, A.T.S., Streck, E.L., Worm, P., Wajner, M., Ritter, F., Netto, C.A., 2000. Preconditioning prevents the inhibition of Na^+,K^+-ATPase activity after brain ischemia. Neurochemical Research 25**,** 969 – 973.

Wyse, A.T., Zugno, A.I., Streck, E.L., Matté, C., Calcagnotto, T., Wannmacher, C.M., Wajner, M., 2002. Inhibition of $Na(^+),K(^+)$-ATPase activity in hippocampus of rats subjected to acute administration of homocysteine is prevented by vitamins E and C treatment. Neurochemical Research 27, 1685-1689.

Zaidi, S.M., Banu, N., 2004. Antioxidant potential of vitamins A, E and C in modulating oxidative stress in rat brain. Clinica Chimica Acta 340, 229-33.

Zarkovic, K., 2003. 4-hydroxynonenal and neurodegenerative diseases. Molecular Aspects in Medicine 24, 293-303. Review.

Zhang, X.L., Jiang, B., Li, Z.B., Hao, S., An, L.J., 2007. Catalpol ameliorates cognition deficits and attenuates oxidative damage in the brain of senescent mice induced by D-galactose. Pharmacology Biochemistry Behavioral 88, 64-72.

Zugno, A.I., Scherer, E.B., Mattos, C., Ribeiro, C.A., Wannmacher, C.M., Wajner, M., Wyse, A.T., 2007. Evidence that the inhibitory effects of guanidinoacetate on the activities of the respiratory chain, Na^+,K^+-ATPase and creatine kinase can be differentially prevented by taurine and vitamins E and C administration in rat striatum in vivo. Biochimica et Biophysica Acta 1772, 563-569.

Figure 1. Effect of pretreatment with vitamins E and C on Na^+, K^+ - ATPase activity in synaptic plasma membrane from rat striatum after intrastriatal hypoxanthine injection. Data are means ± SD for four animals in each group. * $p<0.05$ compared to sham group (ANOVA followed by Duncan multiple-range test). Hpx – hypoxanthine; Vit: vitamins E and C.

Figure 2. Effect of pretreatment with vitamins E and C on TBARS and TRAP in rat striatum after intrastriatal hypoxanthine injection. Data are means ± SD for four animals in each group. * $p<0.05$ compared to sham group (ANOVA followed by Duncan multiple-range test). Hpx – hypoxanthine; Vit: vitamins E and C.

Figure 3. Effect of pretreatment with vitamins E and C on SOD (A), CAT (B) and GPx (C) activities in rat striatum after intrastriatal hypoxanthine administration. Data are means ± SD for four-five animals in each group. * $p<0.05$ compared to sham group (ANOVA followed by Duncan multiple-range test). Hpx – hypoxanthine; Vit: vitamins E and C.

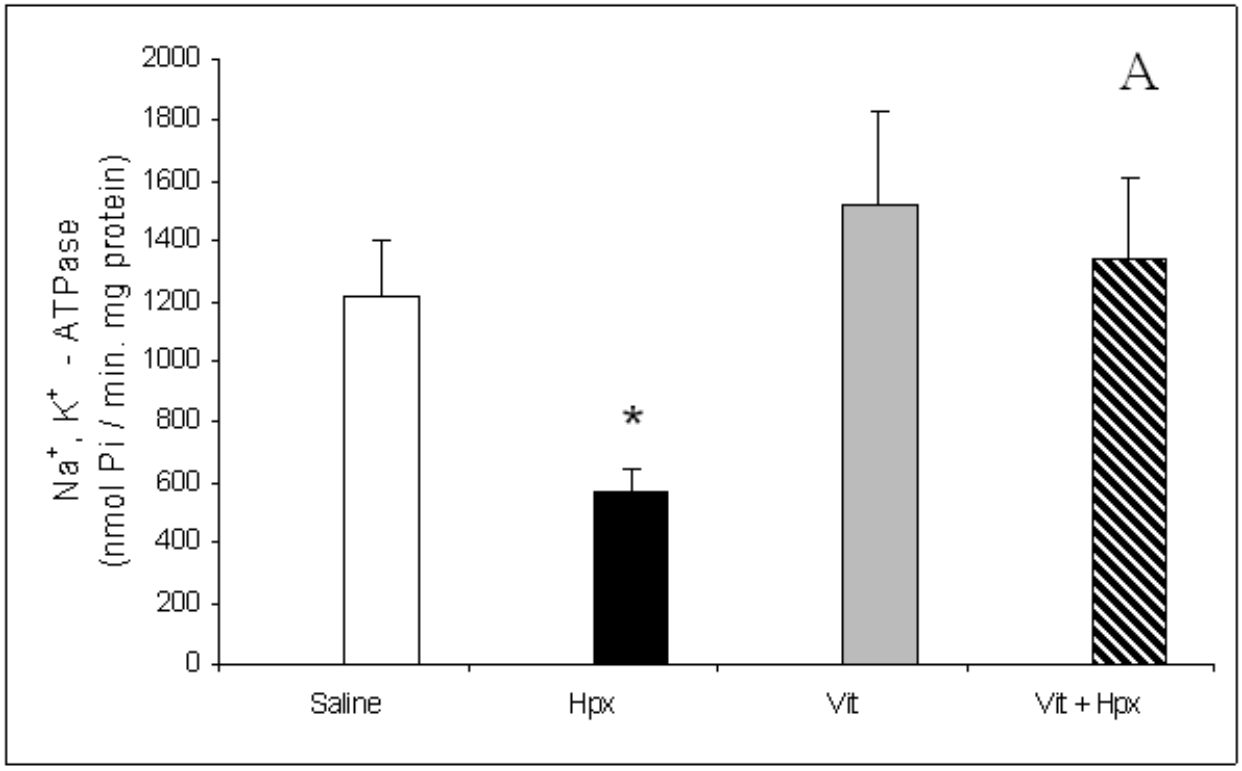

A
2000
1800
1600
1400
1200
1000
800
600
400
200
0
Na+, K+ - ATPase
(nmol Pi / min. mg protein)
*
Saline
Hpx
Vit
Vit + Hpx

Figure 1

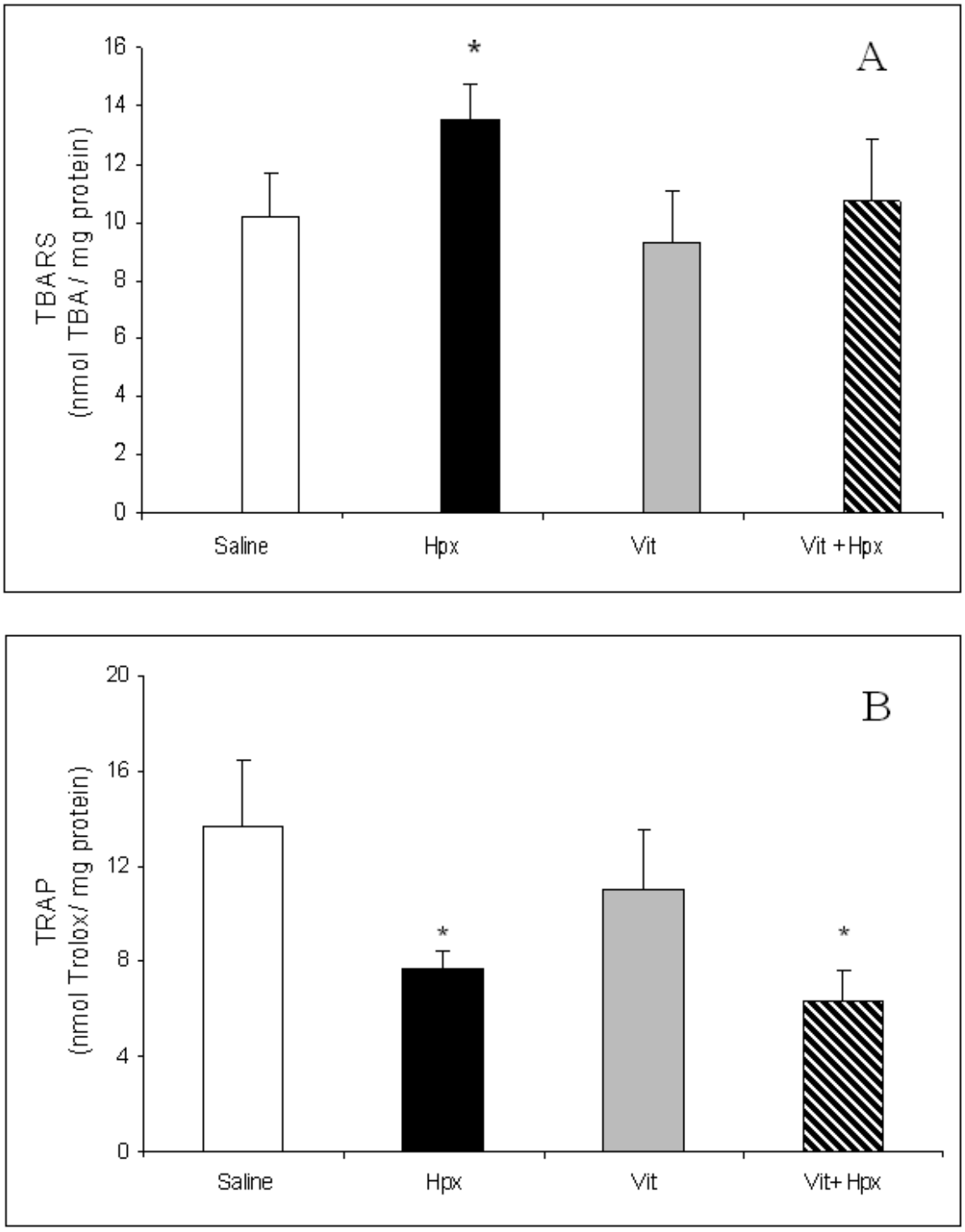
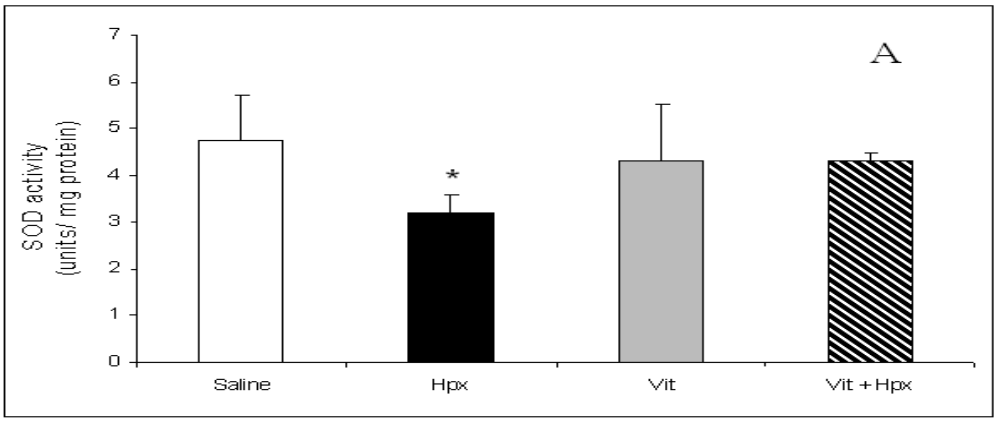
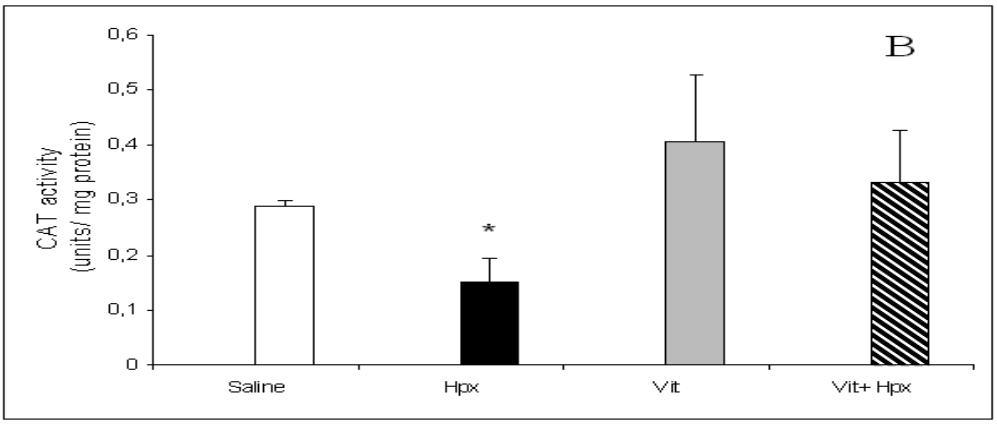

A
TBARS
(nmol TBA / mg protein)
16
14
12
10
8
6
4
2
0
*
Saline
Hpx
Vit
Vit + Hpx
B
TRAP
(nmol Trolox/ mg protein)
20
16
12
8
4
0
*
*
Saline
Hpx
Vit
Vit+ Hpx

Figure 2


A
SOD activity
(units/ mg protein)
7
6
5
4
3
2
1
0
*
Saline
Hpx
Vit
Vit + Hpx


B
CAT activity
(units/ mg protein)
0,6
0,5
0,4
0,3
0,2
0,1
0
*
Saline
Hpx
Vit
Vit+ Hpx

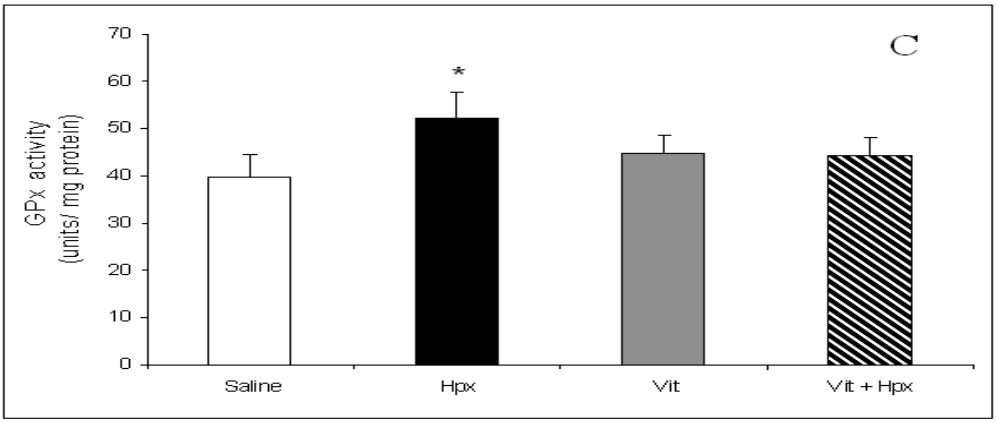

C
GPx activity
(units/ mg protein)
70
60
50
40
30
20
10
0
*
Saline
Hpx
Vit
Vit + Hpx

Figure 3

Capítulo IV – Artigo 04

Intrastriatal injection of hypoxanthine alters striatal ectonucleotidases activities: time-depend effect.

Caren S.Bavaresco, Fabria Chiarani, Janaina Kolling, Denise B. Ramos, Giana P. Cognato, Carla D. Bonan, Maurício R. Bogo, João J. F.Sarkis, Carlos A. Netto and Angela T.S. Wyse

Submetido para publicação na revista Neurochemical Research, 2007

Intrastriatal injection of hypoxanthine alters striatal ectonucleotidases activities: time-depend effect.

Caren S.Bavaresco[1], Fabria Chiarani[1], Janaina Kolling[1], Denise B. Ramos[1], Giana P. Cognato[1], Carla D. Bonan[2], Maurício R. Bogo[2], João J. F.Sarkis[1], Carlos A. Netto[1] and Angela T.S. Wyse[1]

[1]Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil;

[2]Departamento de Biologia Celular e Molecular, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, RS, Brazil.

[1]Address reprint request to: Dr. Angela T.S. Wyse, Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2600-Anexo, CEP 90035-003 Porto Alegre, RS, Brazil, Telephone number: 55 51 33085573;FAX: 55 51 33085535; E-mail: wyse@ufrgs.br.

Abstract

The aim of this study was to investigate the effects of intrastriatal injection of hypoxanthine on ectonucleotidases (E-NTPDases and ecto-5´-nucleotidase) activities in striatum of rats. The expression of these enzymes was studied, as well as, the effect of pre-treatment with vitamins E and C on the effects elicited by this oxypurine on enzymatic activities and on TBARS and the *in vitro* effect of different concentrations of hypoxanthine on ectonucleotidases activities. Adult Wistar rats were divided in (1) control and (2) hypoxanthine-injected group. For ectonucleotidases activities determination, the animals were sacrificed 30 min, 24 h and 7 days after drug infusion. For the evaluation of the expression of NTPDase1-3 and also ecto-5´-nucleotidase, TBARS assay and influence of the pretreatment with vitamins on ectonucleotidases activities, the animals were sacrificed 24 h after hypoxanthine infusion. Results show that hypoxanthine infusion significantly inhibited ectonucleotidases activities and increased TBARS only 24h after this oxypurine administration and the pretreatment with vitamins was able to prevent those effects. Moreover, ecto-5´-nucleotidase expression was increased (80%) 24 h after hypoxanthine infusion; however hypoxanthine *in vitro* did not alter nucleotide hydrolysis. We suggest that these biochemical modifications induced by hypoxanthine could be involved in the pathophysiology of Lesch Nyhan disease.

Key Words: Lesch-Nyhan disease; Intrastriatal hypoxanthine administration; ectonucleotidases; oxidative stress; NTPDase.

5. Introduction

Lesch Nyhan disease is an X-linked hereditary disorder caused by deficiency of hypoxanthine-guanine phosphoribosyltranspherase (HPRT) activity [1-4] in which occur tissue accumulation of hypoxanthine. Affected patients present cognitive deficits, hyperuricemia, spasticity, dystonia and self-mutilation behavior, which is characterized by biting of the lips, tongue and fingers with apparent tissue loss [4-7] In addition, patients present a prominent loss of striatal dopamine [4].

Although the connection between neurological and behavioral dysfunctions present in Lesch Nyhan disease and altered purine metabolism has not been elucidated, tissue accumulation of oxypurines such as hypoxanthine has been proposed to contribute to the neurological dysfunction present in this disease [8-11]. In this scenario, a recent study demonstrated that intrastriatal administration of hypoxanthine significantly inhibited Na^+, K^+-ATPase, AChE and catalase activities in a time-dependent manner [12]. Moreover, it has been demonstrated that hypoxanthine/xanthine oxidase acts as a source of oxidative stress in the vascular system [13] and might contribute to the destruction of blood-brain barrier observed in ischemic brain tissue [14]. A previous study from our group demonstrated that hypoxanthine *in vitro* increased TBARS and reduced TRAP in rat striatum, suggesting an induction of oxidative stress [15].

It has been shown that some purines are recognized as neuromodulators in different brain areas. The biological role of ATP is not only as metabolic fuel since ATP hydrolysis products (ADP, AMP and adenosine) could act as signaling molecules [16]. In this context, some studies showed that adenosine exerted a regulatory mechanism on monoamines system [17,18] and this purine has been involved in motor and behavioral changes through it action in specific receptors named A_1, A_2, A_{2B} and A_3 [19,20]. Beside

this, evidence in the literature point to the involvement of adenosine receptor in aggressive behavior after clonidine administration in mice [21]. Extracellular concentrations of adenosine depend on a regulatory mechanism between the release of this compound from the intracellular medium and the catabolism of ATP, ADP and AMP by ectonucleotidase, such as NTPDases (nucleoside triphosphate diphosphohydrolase) and ecto-5´-nucleotidase (CD73) [22].

Ectonucleotidases are membrane bound enzymes with catalytic site located in the extracellular medium responsible for ATP and ADP breakdown and have been involved in modulation on synaptic transmission, adult neurogenesis and thromboregulation [22]. Moreover, ecto-5´-nucleotidase is a surface-located ecto-enzyme, anchored to the plasmatic membrane via glycosyl phosphatidyinositol (GPI) anchor and it is responsible for extracellular hydrolysis of AMP to adenosine [23].

It has been suggested that a deficit on adenosine-mediated neuronal modulation could be involved in the pathological basis of Lesch Nyhan disease [24]. In this context, Torres and colleagues [25] demonstrated that hypoxanthine altered adenosine transport in peripheral blood lymphocytes from both control and Lesch-Nyhan patients. Beside this, Pesi and colleagues [26] showed that cytosolic 5´-nucleotidase activity was increased in erythrocytes from individuals with Lesch Nyhan disease. Moreover, urinary excretion of adenosine was decreased in patients with Lesch Nyhan disease [27].

In the present study we investigated the effect of intrastriatal hypoxanthine injection on ectonucleotidase activities. We also determined the relative expression of ectonucleotidases in rat striatum. The effect of the pretreatment with vitamins E and C on the effects mediated by hypoxanthine on ectonucleotidases and thiobarbituric acid reactive species (TBARS), as well as the *in vitro* effect of this oxypurine on these

enzymes were also evaluated. The drug was infused in striatum because patients with this syndrome present characteristic alterations in the basal ganglia [4].

6. Materials and Methods

2.1. Animals and reagents

Sixty-days-old male Wistar rats were obtained from the Central Animal House of the Department of Biochemistry, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil. Animals were maintained on a 12 h light/dark cycle (lights on from 7 a.m. to 7 p.m.) in one air-conditioned constant temperature (22^{o} C) colony room, with free access to a 20% (w/w) protein commercial chow and water. Animal care followed the official governmental guidelines in compliance with the Federation of Brazilian Society for Experimental Biology and was approved by ethics committee of the Federal University of Rio Grande do Sul, Brazil and followed the NIH Guide for the Care and Use of Laboratory Animals (NIH publication 85-23, revised 1985). All chemicals were purchased from Sigma Chemical Co., St Louis, MO, USA.

2.2. *In vivo studies*

2.2.1. Experimental treatment

For stereotaxic surgery and cannula placement, rats were anesthetized with ketamine and xilazine (75 and 10 mg/kg ip, respectively) and placed in a rodent stereotaxic apparatus. Under stereotaxic guidance, a 27-gauge stainless cannula (0.9 mm O.D.) with an inner needle guide was inserted unilaterally into the right striatum (coordinates relative from bregma: AP, - 0,5 mm; ML - 2,5 mm; V - 2,5 mm from the dura) [28]. Two days after the surgery, a 30–gauge needle was inserted into the guide

cannula in order to inject buffered hypoxanthine (10 μM) or vehicle (saline) into the right striatum over a 1 min interval. The volume administered (saline or hypoxanthine) was 2μL. Animals were divided into two groups: group 1 (vehicle group), rats that received intrastriatal saline and group 2 (hypoxanthine treated), rats that received intrastriatal hypoxanthine solution (20 pmol/2μL). Hypoxanthine concentration was chosen according to Puig and colleagues [29]. For nucleotidases activities assays, rats were sacrificed 30 min, 24 h or 7 days after drug infusion. For the analysis of gene expression by semi-quantitative RT-PCR, TBARS and for the measurement of nucleotidases activities assays after the pre-treatment with vitamins E and C animals were sacrificed 24 h after hypoxanthine or vehicle (saline) injections.

2.3 Tissue preparation

Animals were killed by decapitation without anesthesia, the brain was removed and striatum were dissected out. For nucleotidases assay, brains were placed in ice-cold isolation medium (320 mM sucrose, 5.0 mM HEPES, pH 7.5, and 0.1 mM EDTA), and striatum was immediately dissected on ice. The striatum was gently homogenized in 5 volumes of ice-cold isolation medium with a motor-driven Teflon-glass homogenizer and synaptosomes were isolated as previously described [30].

2.4. Synaptosomes preparation

Synaptosomes were isolated as previously described [30]. Briefly, 0.5 ml of the crude mitochondrial fraction was mixed with 4.0 ml of an 8.5% Percoll solution and layered onto an isoosmotic Percoll/sucrose discontinuous gradient (10/16%). The synaptosomes that banded at the 10/16% Percoll interface were collected with wide tip disposable plastic transfer pipettes. The synaptosomal fractions were washed twice at 15,000 g for 20 min with the same ice-cold medium to remove the contaminating Percoll, and the synaptosome pellet was then resuspended to a final protein concentration of approximately 0.5 mg/ml. The material was prepared fresh daily and maintained at 0-4°C throughout preparation. The synaptosomal fraction was used for assays immediately after the preparation.

2.5. Determination of NTPDase and 5'-Nucleotidase Activities in Synaptosomes of striatum.

The reaction medium used to assay ATP and ADP hydrolysis was essentially as previously described [31] and contained 5.0 mM KCl, 1.5 mM $CaCl_2$, 0.1 mM EDTA, 10 mM glucose, 225 mM sucrose, and 45 mM Tris-HCl buffer, pH 8.0, in a final volume of 200 µl.

The reaction medium used to assay 5'-nucleotidase activity contained 10 mM Mg Cl_2, 100 mM Tris-HCl, pH 7.5, and 0.15 M sucrose in a final volume of 200 µl [32].

The synaptosome preparation (10-20µg protein) was added to the reaction medium and pre-incubated for 10 min at 37°C. The reaction was initiated by the addition of ATP, ADP or AMP to a final concentration of 1.0 mM and stopped by the addition of 200 µl trichloroacetic acid (10%). The samples were chilled on ice for 10

min, and 100 µl samples were taken for the assay of released inorganic phosphate (Pi) using a colorimetric method [33]. Incubation times and protein concentration were chosen in order to ensure the linearity of the reactions. Controls with the addition of the enzyme preparation after addition of trichloroacetic acid (final concentration 5%) were used to correct nonenzymatic hydrolysis of the substrates. All samples were run in triplicate. Enzyme activities were expressed as nanomoles of Pi released per minute per milligram of protein.

2.6. Analysis of gene expression by semi-quantitative RT-PCR

The analysis of the expression of NTPDase1, NTPDase2, NTPDase3 and ecto-5´-nucleotidase was carried out by a semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) assay. Twenty-four hours after intrastriatal injection of hypoxanthine or vehicle (saline), the striatum of rats was isolated for total RNA extraction with Trizol reagent (Invitrogen) in accordance with the manufactured instructions. The cDNA species were synthesized with Super Script First-Strand Synthesis System for RT-PCR (Invitrogen) from 3 µg of total RNA and oligo (dT) primer in accordance with the suppliers. RT reactions were performed for 50 min at 42°C. cDNA (0.1µl) was used as a template for PCR with specific primers for NTPDase1, NTPDase2, NTPDase3 and ecto-5´-nucleotidase. ß-actin PCR was performed as a control for cDNA synthesis. PCR reactions were performed (total volume of 25 µl) using a concentration of 0.4 µM of each polymerase (Invitrogen) in the supplied reaction buffer.

Conditions for all PCRs were as follow: Initial 1 min denaturation step at 94°C, 1 min at 94°C, 1 min annealing step (NTPDase1, NTPDase3 and ecto-5´-nucleotidase: 65°C; NTPDase2: 66°C; ß-actin: 58.5 °C), 1 min extension step at 72°C for 35 cycles

and a final 10 min extension at 72ºC. The amplification products were: NTPDase1 - 543bp; NTPDase2 - 331bp; NTPDase3 - 267bp; ecto-5´-nucleotidase - 405 bp; ß-actin - 210bp. PCR products were submitted to electrophoresis using a 1% agarose gel. Bands intensities were analyzed by Kodak 1D v.3.5.4 soft-ware. The following set of primers were used: for NTPDase1: 5´-GAT CAT CAC TGG GCA GGA GGA AGG-3´ and 5 ´AAG ACA CCG TTG AAG GCA CACA TGG-3´; for NTPDase2: 5´-GCT GGG TGG GCC GGT GGA TAC G-3´ and 5´ATT GAA GGC CCG GGG ACG CTG AC-3 ´; for NTPDase3: 5´-CGG GAT CCT TGC TGT GCG TGG CAT TTC TT-3´and 5 ´-TCT AGA GGT GCT CTG GCA GGA ATC AGT-3´; for ecto-5´-nucleotidase: 5 ´-CCC GGG GGC CAC TAG CAC CTC A-3´and 5´-GCC TGG ACC ACG GGA ACC TT-3´; for ß-actin: 5´-TAT GCC AAC ACA GTG CTG TCT GG-3´and 5´-TAC TCC TGC TTC CTG ATC CAC AT-3´.

2.7. Pretreatment with vitamins E and C

In this set of experiments, the animals were pre-treated daily during 7 days with intraperitonial administration of alpha-tocopherol (40 mg/kg) and ascorbic acid (100 mg/kg). Controls animals received saline. Doses of vitamins E and C were chosen according Wyse and colleagues [34].

2.8. TBARS (thiobarbituric acid reactive species)

To determinate the lipid peroxidation, we measured the formation of the thiobarbituric acid reactive species (TBARS) as described by Esterbauer and Chessman [35]. TBARS were determined by the absorbance at 535 nm.

2.9. In vitro studies

In order to evaluate the *in vitro* effect of hypoxanthine on nucleotide hydrolysis, different concentrations of hypoxanthine (1.7, 7.0 and 10 μM) were added to the preincubation medium of enzymatic assay. Hypoxanthine concentration was chosen according to Puig and colleagues [29] similar to the concentrations found in plasma from Lesch Nyhan patients. Controls did not contain hypoxanthine.

2.10. Protein determination

Protein was measured by the Coomassie Blue method, according to Bradford [36] using bovine serum albumin as standard.

2.11. Statistical analysis

Data were analyzed by Student´s t-test or one-way analysis of variance (ANOVA) followed by the Duncan multiple range tests when the F-test was significant. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software in a PC compatible computer. Values of $P<0.05$ were considered to be significant.

3.0. Results

3.1. Experiment 1: Effect of intrastriatal hypoxanthine infusion on ATP, ADP and AMP hydrolysis from synaptosomes of striatum of rats.

Figure 1 shows the effect of intrastriatal injection of hypoxanthine on nucleotide hydrolysis in striatum of rats at different periods after oxypurine administration. As can be seen, ATP [$t(6)=3.200$; $p<0.01$] (Fig. 1A), ADP [$t(6)=2.828$; $p<0.01$] (Fig. 1B) and AMP [$t(6)=3.551$; $p<0.01$] (Fig. 1C) hydrolysis were significantly inhibited 24 h after hypoxanthine infusion, but not 30 min {ATP [$t(6)=0.739$; $p>0.05$] (Fig. 1A); ADP

[t(6)=1.395; p>0.05] (Fig. 1B); AMP [t(6)=0.563; p>0.05] (Fig. 1C)} or 7 days {ATP [t(6)=1.042; p>0.05] (Fig. 1A); ADP [t(6)=0.695; p>0.05] (Fig. 1B); AMP [t(6)=0.543; p>0.05] (Fig. 1C)}. Nucleotide hydrolysis was not altered after hypoxanthine administration in contralateral striatum (data not shown).

3.2. Experiment 2: Relative expression of striatal NTPDases and ecto-5´-nucleotidase analyzed by semi-quantitative RT-PCR after 24 hours of intrastriatal hypoxanthine.

Since nucleotides hydrolysis was only inhibited 24 h after hypoxanthine administration, we also analyzed the relative expression of NTPDases (NTPDase1, NTPDase2 and NTPDase3) and ecto-5´-nucleotidase in rat striatum after 24 h of hypoxanthine administration. As can be seen on Figure 2, the relative expression of NTPDase 1 (Fig. 2 A-B), NTPDase 2 (Fig. 2 C-D), and NTPDase 3 (Fig. 2 E-F), were not altered by hypoxanthine treatment. However, relative expression of ecto-5 ´-nucleotidase (Fig. 2 G-H) was significantly increased (80%) after oxypurine administration.

3.3. Experiment 3: Effect of hypoxanthine on TBARS in rat striatum 24 h after intrastriatal hypoxanthine infusion.

The effect of 24h intrastriatal injection of hypoxanthine on TBARS, an index of lipid peroxidation, in striatum of rats was also studied. As can be seen in figure 3, hypoxanthine infusion was able to increase TBARS in striatum of rats [t(10)=2.534; p<0.05]

3.4. Experiment 4: Effect of the pretreatment with vitamins E and C on the *ectonucleotidase* activities *and on TBARS in* rat striatum 24 h after *intrastriatal hypoxanthine infusion.*

In this set of experiments, the effect of pretreatment with vitamins E and C on the effects elicited by 24 h intrastriatal injection of hypoxanthine on nucleotides hydrolysis in striatum of rats. Results revealed that pretreatment with vitamins E and C prevents the inhibitory effect on ATP, ADP and AMP hydrolysis mediated by hypoxanthine infusion [$F(3,12)=4.425; p< 0.05$] (Figure 4).

Moreover, we also verified the effect of vitamins E and C administration on the lipid peroxidation caused by hypoxanthine infusion on TBARS assay. As can be seen in Figure 5, pretreatment with this antioxidants was able to prevent TBARS increased elicit by hypoxanthine administration [$F(3,16)=3.395; p< 0.05$]

3.5. Experiment 5: In vitro effect of hypoxanthine on ATP, ADP and AMP hydrolysis from synaptosomes of striatum of naïve rats.

In order to evaluate the direct effect of hypoxanthine on nucleotides hydrolysis, we tested the *in vitro* effect of different concentrations of hypoxanthine (1.7, 7.0 and 10μM) on ATP (Fig. 6A), ADP (Fig. 6B) and AMP (Fig. 6C) hydrolysis from striatal synaptosomes of rats. Results demonstrated that the addition of hypoxanthine to the enzymatic assay was not able to alter nucleotides hydrolysis in all tested concentrations {ATP [$F(3,12)=0.543; p>0.05$]; ADP [$F(3,12)=0.492; p>0.05$]; AMP [$F(3,12)=0.543; p>0.05$]}.

Discussion

Lesch Nyhan disease is an X-linked hereditary disorder caused by deficiency of hypoxanthine-guanine phosphoribosyltranspherase activity. Affected patients present hyperuricemia, spasticity, dystonia, mental retardation and self-mutilation behavior [6],

along with a dysfunction of the dopamine transmitter system of the basal ganglia [4]. A common feature of Lesch Nyhan is that patients exhibit characteristically raised level of hypoxanthine in plasma [37-39] urine [38] and cerebrospinal fluid [37-39]. However, the pathophysiology of this disease is still obscure.

NTPDases form a group of enzymes that can hydrolyze ATP and ADP to AMP with different abilities and the 5'-nucleotidase promotes the hydrolysis of AMP to adenosine. Since ATP could act as an excitatory neurotransmitter, deregulation on the delicate control of ATP hydrolysis could be involved in some neuronal diseases [40,41]. In this scenario, NTPDases and ecto-5´-nucleotidase activities could be inhibited throughout a stoichiometric mechanism promoted by ATP and ADP hydrolysis inhibition [42]. In the present study, we firstly evaluated the effect of hypoxanthine administration on ectonucleotidases (NTPDases and ecto-5´-nucleotidase) activities in striatum of rats at different post-infusion times. Results showed that intrastriatal hypoxanthine infusion inhibits the hydrolysis of ATP (35%), ADP (27%) and AMP (29%) 24 h after oxypurine administration but not after 30 min or 7 days. The inhibition of ATP hydrolysis observed in this study was possibly leading to the nucleotide accumulation onto the extracellular medium. In this context, studies showed that larges amounts of ATP in the extracellular medium could mediate cell death thought activation of P2X receptors [43] and it has been involved in the induction of different cell type's death including hepatocytes, microglial and myeloid cells [44,45] At this point, it's important to note that nucleotide hydrolysis returned to basal values 7 days after intrastriatal hypoxanthine infusion. Since ectonucleotidases can also respond by compensatory alterations in their activities due to alteration of gene transcription [42], this modification observed in our study 7 days after hypoxanthine infusion could be a consequence of enzymatic adaptation.

Since the alterations induced by intrastriatal hypoxanthine in NTPDases and ecto-5´-nucleotidase activities could exert modifications in transcriptional control, we decide to investigate the relative expression of NTPDases (NTPDase1, NTPDase2 and NTPDase3) and ecto-5´-nucleotidase in rat striatum after 24 h of hypoxanthine administration. Our results showed that intrastriatal hypoxanthine infusion was able to up-regulate ecto-5´-nucleotidase mRNAs. We did not know the exact mechanism, but a possibility that could explain this phenomenon despite enzymatic inhibition is known as positive feedback autoregulatory loop. Moreover, Braun and colleagues [46] showed that permanent focal ischemia causes a reactive increase in 5´-nucleotidase on astrocytes and microglial cells. On the other hand, Zimmermann and colleagues [23] discussed that this up-regulation on ecto-5´-nucleotidase mRNAs could increases the capacity of the tissue to hydrolyze extracellular AMP possibly derived from ATP liberated by damaged tissue and, consequently, in this case, adenosine could exert neuroprotective effect. This interpretation is in agreement with other authors in the literature [42,47].

Neychev and Mitev [48] proposed that modifications on adenosine system could implicate in SNC alterations such as self-injuries, similar to those observers in Lesch Nyhan disease. Moreover, Torres et al. [25] suggested that the excess of hypoxanthine in striatum could alter extracellular adenosine concentration leading to self-injurious biting. Beside this, it has been showed that increased hypoxanthine levels influence equilibrative adenosine transporters in brain [24].

In the present study, we also evaluated some possible mechanisms which could be involved in the inhibition of ectonucleotidases caused by hypoxathine infusion. In this context, our results demonstrated that intrastriatal infusion of hypoxanthine increased TBARS, an index of lipid peroxidation, in rat striatum. On the other hand, pre-treatment with vitamins E and C prevent the effects of intrastriatal hypoxanthine infusion on

nucleotide hydrolysis and on TBARS in rat striatum. On the other hand, we also evaluated the *in vitro* effect of hypoxanthine on ectonucleotidases and results showed that this oxypurine did not alter nucleotide hydrolysis when added to the incubation medium which could confirm that this oxypurine was possible acting throughout indirect routes, probably by lipid peroxidation and/or oxidative stress. These results are in agreement with other studies from literature pointing to the participation of oxidative stress in the inhibition of NTPDase and ecto-5´-nucleotidase activities in renal epithelial cells and in the brain [41,49]. It has been demonstrated that brain is highly susceptible to oxidative stress because it has low cerebral antioxidant defenses compared to other tissues [50,51] a fact that makes it more vulnerable to increases in reactive oxygen species.

We have shown that hypoxanthine induces free radical generation (enhanced chemiluminescence) and reduces the antioxidant defenses (decreased TRAP), i.e., elicits oxidative stress in the brain. In the present studied, we demonstrated that intrastriatal hypoxanthine infusion increased TBARS in rat striatum and this effect could be preventing by vitamins E and C administration group [12,15,52]. It has been described that vitamin E is a lipid-soluble vitamin that interact with cell membranes, traps free radicals and interrupts oxidative damage, preventing lipid peroxidation and requires vitamin C (ascorbate) for its regeneration [53]. In this scenario, Vietta and colleagues [54] demonstrated that trolox, water soluble vitamin E, was able to totally prevent lipid peroxidation and ADPase activity inhibition caused by oxidant generating system in synaptic plasma membrane. In our study, pretreatment with vitamins E and C could prevent the propagation of lipid peroxidation also acting as a membrane stabilizer in which ectonucleotidases were inserted.

Although it is difficult to extrapolate our results to the human condition, it is conceivable that the inhibition ectonucleotidases activities might be involved in the pathophysiology of the neurological features present in patients with Lesch-Nyhan disease. However, more studies are necessary to investigate other mechanisms involved in this metabolic condition.

Acknowledgements

This work was supported in part by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq – Brazil) and Programa de Núcleos de Excelência-Financiadora de Estudos e Projetos (PRONEX-FAPERGS-CNPq-Brazil).

References

1. Nyhan WL, Oliver WJ, Lesch M (1965) A familial disorder or uric acid metabolism and central nervous system function II. J. Pediatr. 67:439 -444.
2. Henderson JF, (1968) Possible functions of hypoxanthine-guanine phosphoribosyltransferase and their relation to the biochemical pathology of the Lesch-Nyhan syndrome. Fed. Proc. 27:1075-1077.
3. Rijksen G, Staal GEJ, Van der Vlist MJM, et al (1981) Partial hypoxanthine-guanine phosphoribosyl transferase deficiency with full expression of the Lesch – Nyhan syndrome. Hum. Gen. 57:39 – 47.

4. Jinnah HA, Friedmann T, (2001) Lesch Nyhan disease and it variants. In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds), The Metabolic and Molecular Bases of Inherited Disease. McGraw-Hill, New York, pp 2537-2569.
5. Mizuno T, (1986) Long-term follow-up of ten patients with Lesch-Nyhan syndrome. Neuropediatrics 17:158-161.
6. Jinnah HA, Gage FH, (1990) Animal models of Lesch-Nyhan syndrome. Brain Res. Bull. 25:467 – 475.
7. Matthews WS, Solan A, Barabas G, Robey K, (1999) Cognitive functioning in Lesch-Nyhan syndrome: a 4-year follow-up study. Dev. Med. Child. Neurol. 41:260-262.
8. Dasheiff RM, (1980) Benzodiazepinic treatment for Lesch-Nyhan syndrome? Dev. Med. Child. Neurol. 22: 101 – 102.
9. Kisch SJ, Fox IH, Kapur BM, et al (1985) Brain benzodiazepine receptor binding and purine concentration in Lesch-Nyhan. Brain Res. 336:117-123.
10. Visser JE, Bär PR, Jinnah, HA, (2000) Lesch-Nyhan disease and the basal ganglia. Brain Res. Bull. 32:449 – 475.
11. Ma MHY, Stacey NC, Connolly GP, (2001) Hypoxanthine impairs morphogenesis and enhances proliferation of a neuroblastoma model of Lesch Nyhan syndrome. J. Neurosci. Res. 63:500 – 508.
12. Bavaresco CS, Chiarani F, Wannmacher CMD, et al (2007) Intrastriatal hypoxanthine reduces Na^+,K^+ - ATPase activity and induces oxidative stress in the rats. Metab. Brain Dis. 22:1-11. .
13. Oliveira PJ, Rolo AP, Palmeira CM, et al (2001) Carvedilol reduces mitochondrial damage induced by hypoxanthine/xanthine oxidase: relevance to hypoxia/reoxygenation injury. Cardiovasc. Toxicol. 1:205-213.

14. Beckman JS, Liu TH, Hogan EL, et al (1987) Oxygen free radicals and xanthine oxidase in cerebral ischemic injury in the rat. Soc. Neurosci. Abstr. 13:1498.

15. Bavaresco CS, Chiarani F, Matté C, et al (2005) Effect of hypoxanthine on Na^+,K^+-ATPase activity and some parameters of oxidative stress in rat striatum. Brain Res. 1041:198 – 204.

16. Komoszynski M, Wojtczak A, (1996) Apyrases (ATP diphosphohydrolases, EC 3.6.1.5): function and relationship to ATPases. Biochim Biophys Acta. 1310:233-241.

17. Schiffmann SN, Vanderhaeghen JJ, (1993) Age-related loss of mRNA encoding adenosine A2 receptor in the rat striatum. Neurosci Lett. 158: 121-124.

18. Zhu CB, Steiner JA, Munn JL, et al (2007) Rapid stimulation of presynaptic serotonin transport by a3 adenosine receptors. J. Pharmacol. Exp. Ther. 322:332-340.

19. Ribeiro JA, Lobo MG, Sebastião AM. (2003) Endogenous adenosine modulation of 22Na uptake by rat brain synaptosomes. Neurochem Res. 28:1591-1595.

20. Martinelli A, Tuccinardi T, (2007) Molecular modeling of adenosine receptors: new results and trends. Med. Res. Rev. epud ahead of print.

21. Ushijima I, Katsuragi T, Furukawa T. (1984) Involvement of adenosine receptor activities in aggressive responses produced by clonidine in mice. Psychopharmacology (Berl) 83: 335-339.

22. Zimmermann H (2006) Ectonucleotidases in the nervous system. Novartis Found Symp. 276: 113-128.

23. Zimmermann H, Braun N, Kegel B, et al (1998) New insights into molecular structure and function of ectonucleotidases in the nervous system. Neurochem. Int. 32:421-425.

24. Prior C, Torres RJ, Puig JG (2006) Hypoxanthine effect on equilibrative and concentrative adenosine transport in human lymphocytes: implications in the phatogenesis of Lesch-Nyhan syndrome. Nucleosides Nucleotides Nucleic Acids. 25: 1065-1069.

25. Torres RJ, DeAntonio I, Prior C, Puig JG. (2004) Adenosine transport in HPRT deficient lymphocytes from Lesch-Nyhan disease patients. Nucleosides Nucleotides Nucleic Acids. 23:1193-1196.

26. Pesi R, Micheli V, Jacomelli G et al (2000) Cytosolic 5'-nucleotidase hyperactivity in erythrocytes of Lesch-Nyhan syndrome patients. Neuroreport. 9:1827-1831.

27. Sweetman L, Nyhan WL (1970) Detailed comparison of the urinary excretion of purines in a patient with Lesch-Nyhan syndrome and a control subject. Biochem.Med. 4:121-134.

28. Paxinos G, Watson C (1986) The rat brain in stereotaxic coordinates. Academic Press, London.

29. Puig JG, Jimenez ML, Mateos FA (1989) Adenine nucleotide turnover in hypoxanthine-guanine phosphoribosyl-transferase: evidence for an increased contribution of purine biosyntheses de novo. Metabolism 38: 410 – 418.

30. Nagy AK, Delgado-Escueta AV (1984) Rapid preparation of synaptosomes from mammalian brain using a non toxic isoosmotic gradient (Percoll). J. Neurochem. 43:1114-1123.

31. Delwing D, Delwing D, Sarkis, JJ, et al (2007) Proline induces alterations on nucleotide hydrolysis in synaptosomes from cerebral cortex of rats. Brain Res. 1149:210-215.

32. Heymann D, Reddington M, Kreutzberg GW (1984) Subcellular localization of 5'-nucleotidase in rat brain. J. Neurochem. 43:971-978

33. Chan KM, Delfert D, Junger KD (1986) A direct colorimetric assay for Ca^{+} -stimulated ATPase activity. Anal Biochem 157:375 – 380.

34. Wyse AT, Zugno AI Streck EL et al (2002) Inhibition of Na(+),K(+)-ATPase activity in hippocampus of rats subjected to acute administration of homocysteine is prevented by vitamins E and C treatment. Neurochem Res. 12:1685-1689.

35. Esterbauer H, Cheeseman KH. (1990) Determination of aldehydic lipid peroxidation products: malonaldehyde and 4-hydroxynonenal. Methods Enzymol. 186:407-421.

36. Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein –die binding. Anal. Biochem. 72:248 – 254.

37. Rosenbloom RM, Henderson JF, Caldwell IC (1967) Inherited disorder of purine metabolism. JAMA 202:175-177.

38. Harkness RA, McCreanor GM, Watts RW (1988) Lesch-Nyhan syndrome and its pathogenesis: purine concentrations in plasma and in urine with metabolite profiles in CSF. J. Inher. Metab. Dis. 11**:** 239 – 252.

39. Puig JG, Mateos FA (1993) The biochemical basis of HGPRT deficiency. In: Gresser U (ed), Molecular genetics, biochemistry and clinical aspects of inherited

disorders of purine and pyrimidine metabolism. Springer-Verlag, New York, pp 12-26.

40. Berti SL, Bonan CD, da Silva FL (2001) Phenylalanine and phenylpyruvate inhibit ATP diphosphohydrolase from rat brain cortex. Int J Dev Neurosci. 19:649-653.
41. Delwing D, Goncalves MC, Sarkis JJ (2005) L-NAME administration prevents the inhibition of nucleotide hydrolysis by rat blood serum subjected to hyperargininemia. Amino Acids. 29:267-272.
42. Vuaden FC, de Paula Cognato G, Bonorino C(2007). Lipopolysaccharide alters nucleotidase activities from lymphocytes and serum of rats. Life Sci. 80:1784-1791.
43. Matute C, Alberdi E, Domercq M et al (2007) Excitotoxic damage to white matter. J Anat. 210:693-702.
44. Tinton SA, Lefebvre VH, Cousin OC (1993) Cytolytic effects and biochemical changes induced by extracellular ATP to isolated hepatocytes. Biochim. Biophys. Acta 1176:1-6
45. Ferrari D, Chiozzi P, Falzoni S et al (1997) ATP- mediated cytotoxicity in microglial cells. Neuropharmacology 36:1295-1301
46. Braun N, Lenz C, Gillardon F, et al (1997) Focal cerebral ischemia enhances glial expression of 5-nucleotidase. Brain Res. 766:213-226.
47. Krishna SM, Katoor J, Balaram P (2006) Down regulation of adhesion protein E-cadherin in Epstein-Barr virus infected nasopharyngeal carcinomas. Cancer Biomark. 1:271-277.
48. Neychev VK, Mitev VI. (2004) The biochemical basis of the neurobehavioral abnormalities in the Lesch-Nyhan syndrome: a hypothesis. Med Hypotheses. 63:131-134.

49. Siegfried G, Amiel C, Friedlander G. (1996) Inhibition of ecto-5'-nucleotidase by nitric oxide donors. Implications in renal epithelial cells. J Biol Chem. 271:4659-4664.

50. Halliwell B (1996) Free radicals, proteins and DNA: oxidative damage versus redox regulation. Biochem. Soc. Trans. 24:1023 – 1027.

51. Floyd RA (1999) Antioxidants, oxidative stress and degenerative neurological disorders. Proc. Soc. Exp. Biol. Med. 222:236 – 245.

52. Bavaresco CS, Zugno AI, Tagliari, B (2004) Inhibition of Na^+, K^+ - ATPase activity in rat striatum by metabolites accumulated in Lesch Nyhan disease. Int. J. Devl. Neurosci. 22:11-17.

53. Zugno AI, Scherer EB, Mattos, C et al (2007) Evidence that the inhibitory effects of guanidinoacetate on the activities of the respiratory chain, Na^+,K^+-ATPase and creatine kinase can be differentially prevented by taurine and vitamins E and C administration in rat striatum in vivo. Biochim Biophys Acta. 1772:563-569.

54. Vietta M, Frassetto SS, Battastini AM et al (1996) Sensitivity of ATPase-ADPase activities from synaptic plasma membranes of rat forebrain to lipid peroxidation in vitro and the protective effect of vitamin E. Neurochem Res. 21:299-304

Figure 1. Effect of intrastriatal hypoxanthine injection on ATP (A), ADP (B) and AMP (C) hydrolysis in striatum of rats at different times after infusion. Data are means ± SD for four animals in each group. * $p<0.05$ compared sham group (Student's *t* test). Hpx – hypoxanthine.

Figure 2. Gene expression patterns 24h after intrastriatal hypoxanthine injection for NTPDase 1 (A and B), NTPDase 2 (C and D), NTPDase 3 (E and F) and 5´-nucleotidase (G and H) and ß-actin in striatum of rats. Data presented were representative from three individual experiments.

Figure 3. Effect of 24 h intrastriatal hypoxanthine injection on TBARS in rat striatum. Data are means ± SD for five animals in each group. * $p<0.05$ compared sham group (Student's *t* test). Hpx – hypoxanthine.

Figure 4. Effect of pretreatment with vitamins E and C on ATP (A), ADP (B) and AMP (C) hydrolysis 24 h after intrastriatal hypoxanthine injection on in striatum of rats. Data are means ± SD for four animals in each group. *$p<0.05$ compared sham group. (ANOVA followed by Duncan multiple test). Hpx – hypoxanthine.

Figure 5. Effect of pretreatment with vitamins E and C on TBARS 24 h after intrastriatal hypoxanthine injection in striatum of rats. Data are means ± SD for five animals in each group. *$p<0.05$ compared sham group. (ANOVA followed by Duncan multiple test). Hpx – hypoxanthine.

Figure 6. Effect of different hypoxanthine concentrations (1.7, 7.0 and 10.0 μM) on ATP (A), ADP (B) and AMP (C) hydrolysis in synaptosome from striatum of naïve rats. Data are means ± SD for four animals in each group. ** $p<0.01$ compared to sham group (ANOVA followed by Duncan multiple test). Hpx – hypoxanthine.

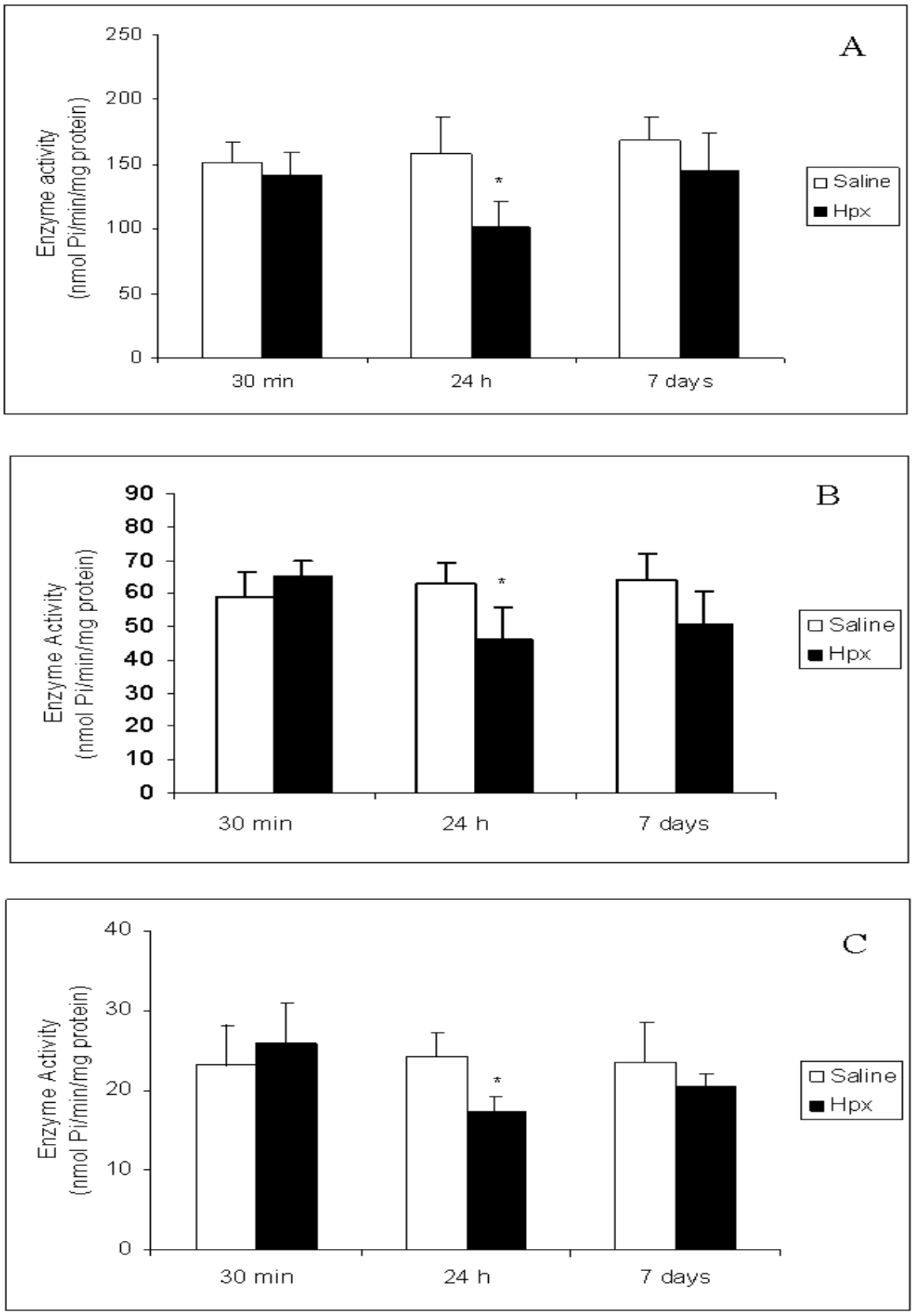

A
Enzyme activity
(nmol Pi/min/mg protein)
250
200
150
100
50
0
30 min
24 h
7 days
*
Saline
Hpx
B
Enzyme Activity
(nmol Pi/min/mg protein)
90
80
70
60
50
40
30
20
10
0
30 min
24 h
7 days
*
Saline
Hpx
C
Enzyme Activity
(nmol Pi/min/mg protein)
40
30
20
10
0
30 min
24 h
7 days
*
Saline
Hpx

Figure 1

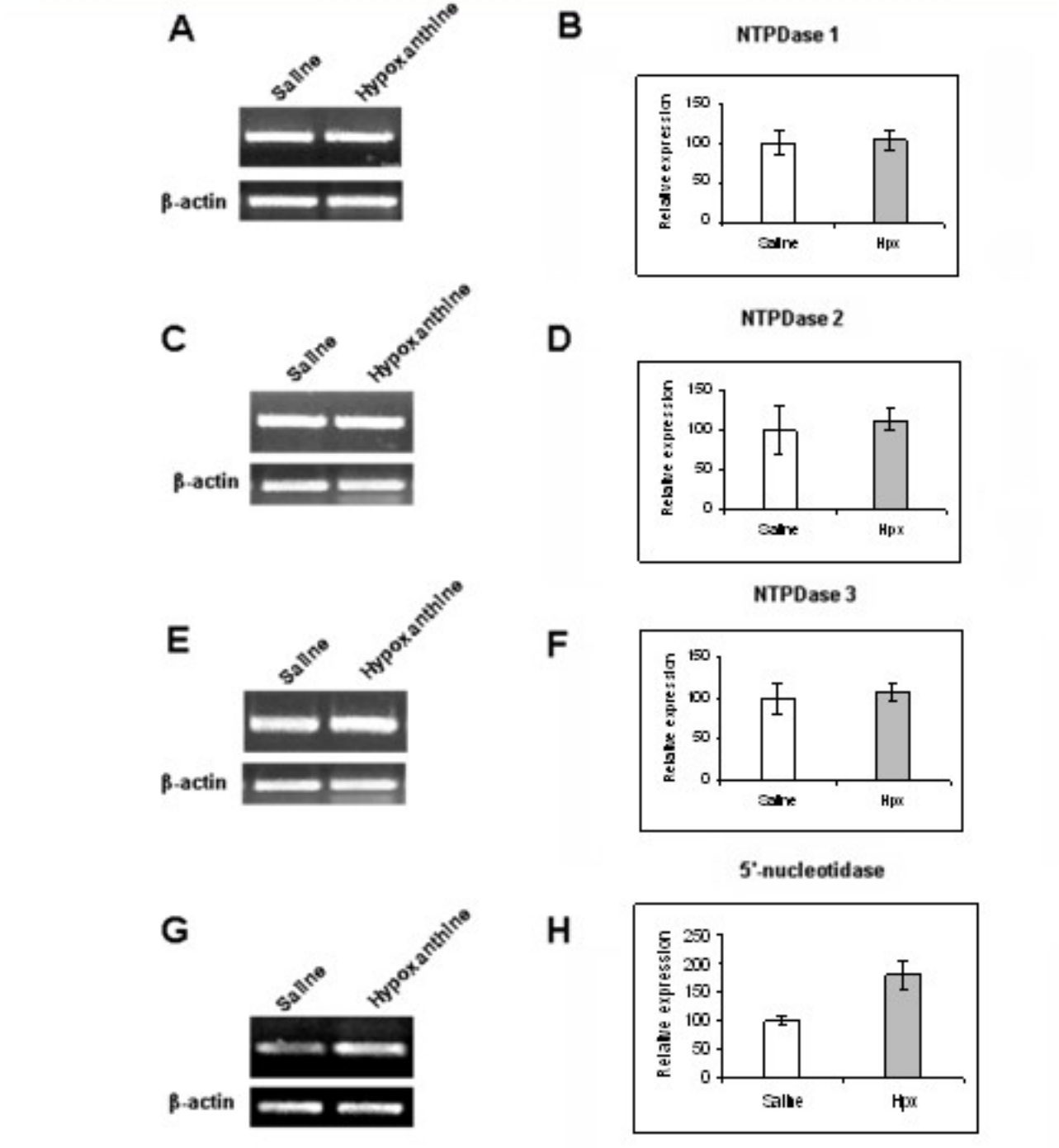

A
Saline
Hypoxanthine
β-actin
B
NTPDase 1
Relative expression
150
100
50
0
Saline
Hpx
C
Saline
Hypoxanthine
β-actin
D
NTPDase 2
Relative expression
150
100
50
0
Saline
Hpx
E
Saline
Hypoxanthine
β-actin
F
NTPDase 3
Relative expression
150
100
50
0
Saline
Hpx
G
Saline
Hypoxanthine
β-actin
H
5'-nucleotidase
Relative expression
250
200
150
100
50
0
Saline
Hpx

Figure 2

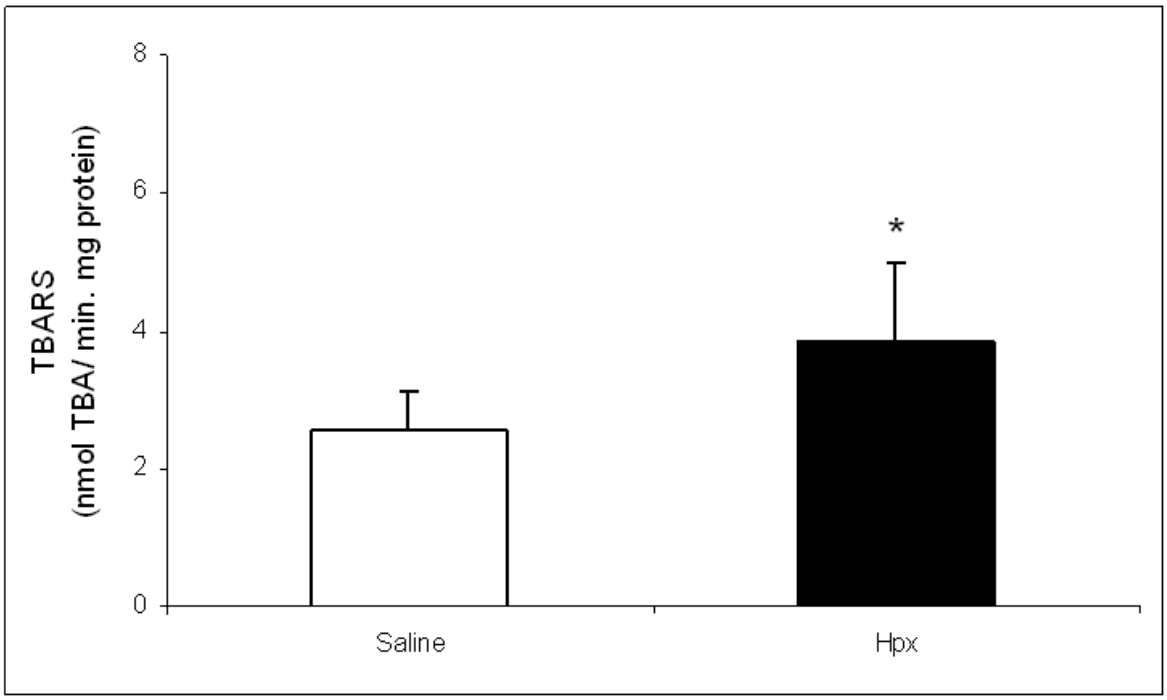


Figure 3

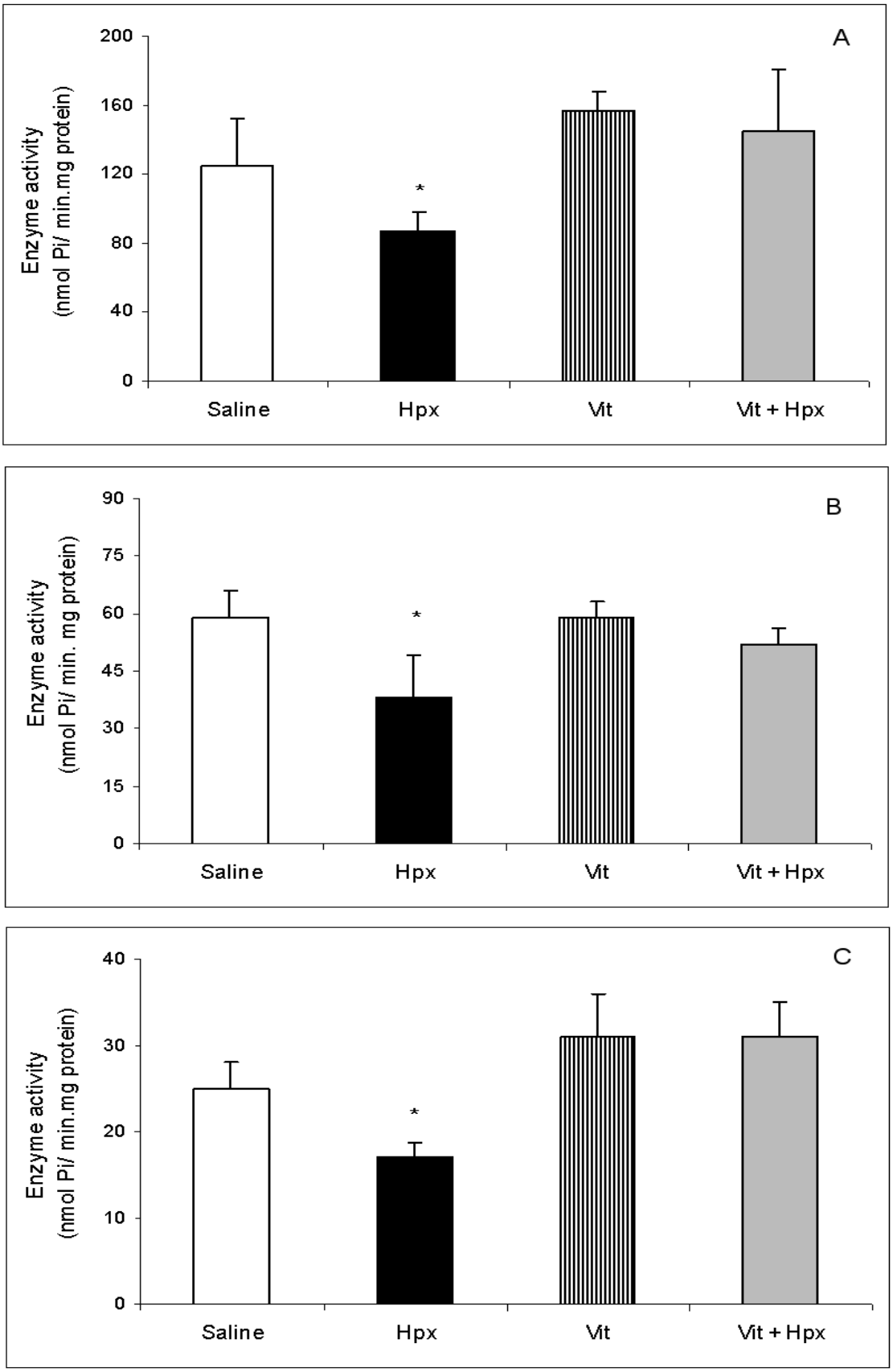

A
Enzyme activity
(nmol Pi/ min.mg protein)
200
160
120
80
40
0
*
Saline
Hpx
Vit
Vit + Hpx
B
Enzyme activity
(nmol Pi/ min. mg protein)
90
75
60
45
30
15
0
*
Saline
Hpx
Vit
Vit + Hpx
C
Enzyme activity
(nmol Pi/ min.mg protein)
40
30
20
10
0
*
Saline
Hpx
Vit
Vit + Hpx

Figure 4

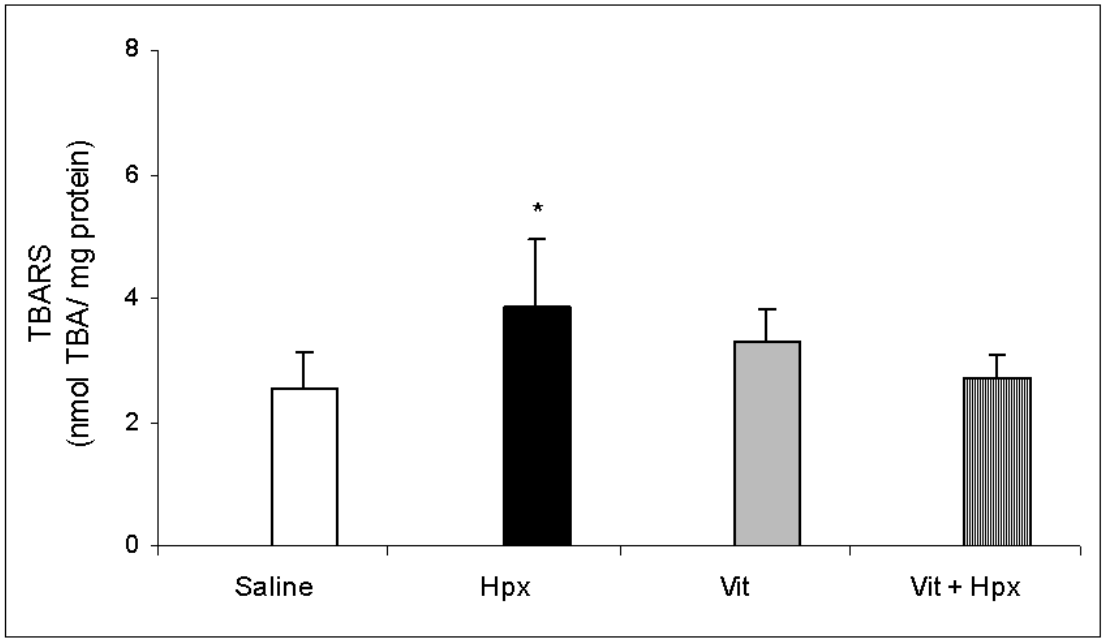
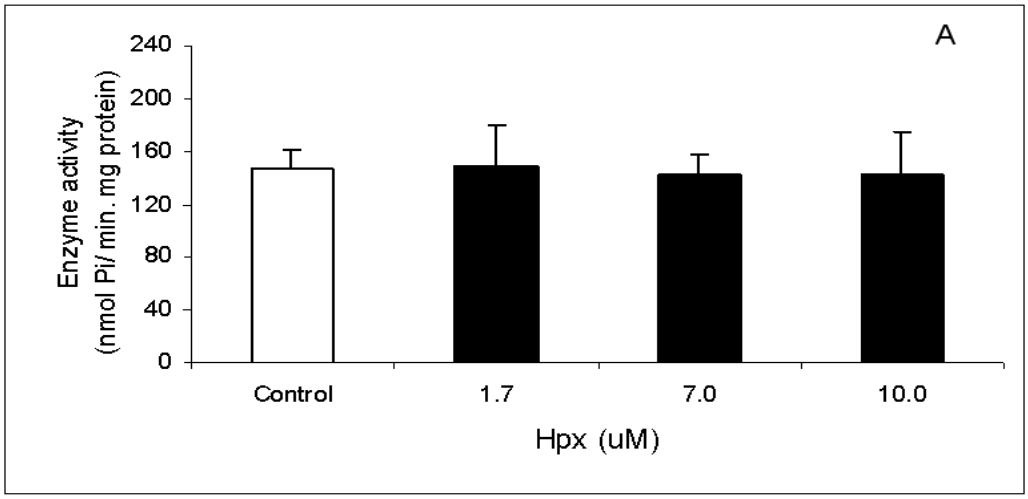
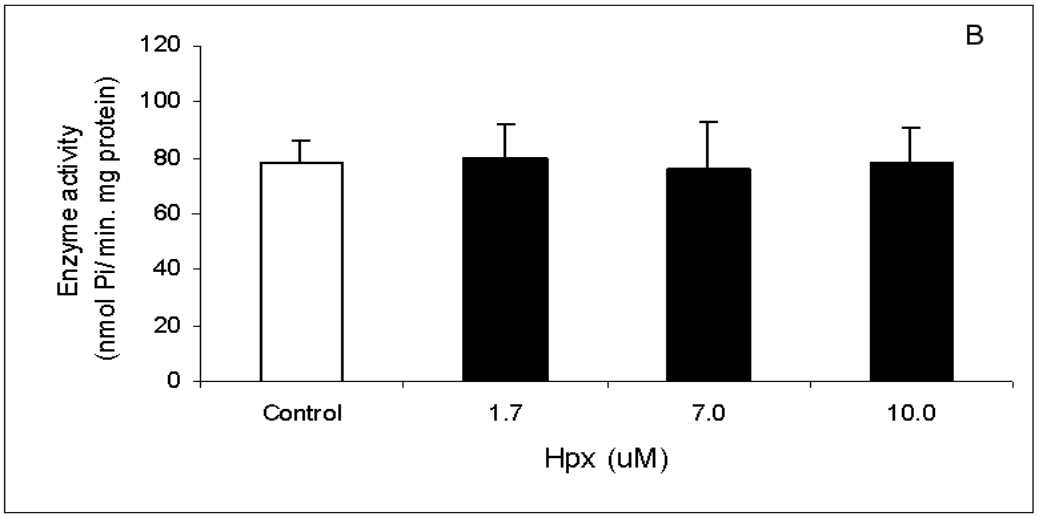


Figure 5


A
Enzyme activity
(nmol Pi/ min. mg protein)
240
200
160
120
80
40
0
Control
1.7
7.0
10.0
Hpx (uM)


B
Enzyme activity
(nmol Pi/ min. mg protein)
120
100
80
60
40
20
0
Control
1.7
7.0
10.0
Hpx (uM)

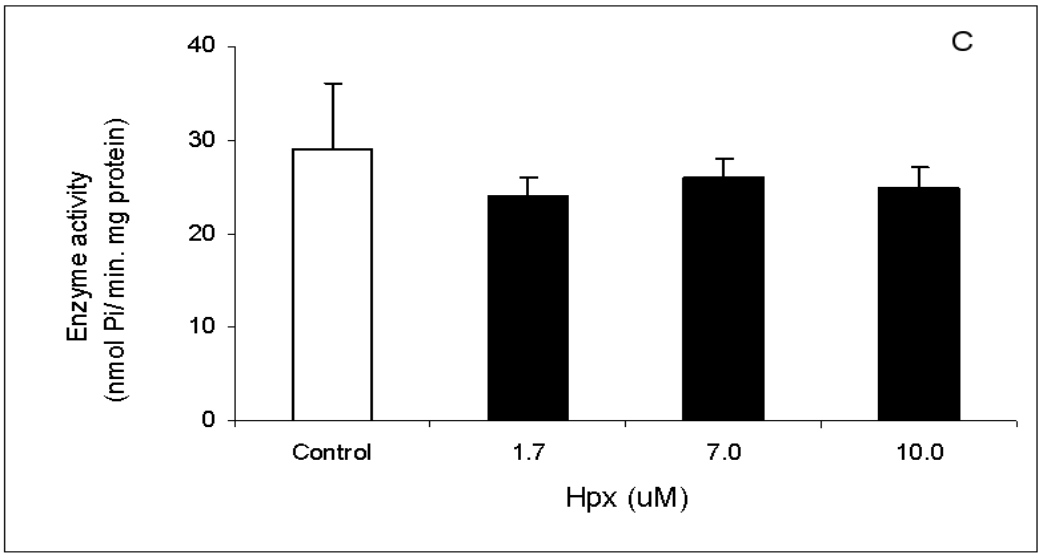

C
Enzyme activity
(nmol Pi/ min. mg protein)
40
30
20
10
0
Control
1.7
7.0
10.0
Hpx (uM)

Figure 6

Capítulo V – Artigo 05

Intrastriatal injection of hypoxanthine reduces striatal serotonin content and impairs spatial memory performance in rats

Caren Serra Bavaresco[1], Fabria Chiarani[1], Eduardo Duringon[1], Marcelo Machado Ferro[2], Cláudio Da Cunha[2], Carlos Alexandre Netto[1] and Angela Terezinha de Souza Wyse[1]

Publicado na revista Metabolic Brain Disease, 2007.

Intrastriatal injection of hypoxanthine reduces striatal serotonin content and impairs spatial memory performance in rats

Caren Serra Bavaresco[1], Fabria Chiarani[1], Eduardo Duringon[1], Marcelo Machado Ferro[2], Cláudio Da Cunha[2], Carlos Alexandre Netto[1] and Angela Terezinha de Souza Wyse[1]

[1]Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil;

[2] Departamento de Farmacologia and Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Paraná, Curitiba, Brazil.

[1]Address reprint request to: Dr. Angela T.S. Wyse, Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2600-Anexo, CEP 90035-003 Porto Alegre, RS, Brazil, Telephone number: 55 51 33165573; E-mail: wyse@ufrgs.br.

Abstract

The aim of this study was to investigate the effects of intrastriatal injection of hypoxanthine, a metabolite accumulated in Lesch-Nyhan disease, on rats performance in the Morris water maze tasks, along with the monoamine content in striatum of rats. Male adult Wistar rats were divided in two groups: (1) saline-injected and (2) hypoxanthine-injected group. Animals were trained in the Morris Water Maze seven days later or were sacrificed for the evaluation of the striatal monoamine content. Results show that hypoxanthine administration caused impairment on spatial navigation in the acquisition phase in reference memory task in the Morris Water Maze, as well as in the latency to cross over the platform location in probe trial, when compared to saline group (control). Hypoxanthine also altered rat performance in the working memory. Although striatal dopamine metabolites content did not change, treated animals showed a reduction of tissue levels of serotonin (5-HT) and 5- hydroxyl-indoleacetic acid (5-HIAA). These results show that intrastriatal hypoxanthine administration provoked impairment of spatial learning/memory in rats without affecting striatal dopaminergic system, although serotonergic pathways seem to have been affected.

Key Words: Lesch-Nyhan disease; Intrastriatal hypoxanthine administration; memory; Serotonin; Dopamine.

7. Introduction

Tissue accumulation of hypoxanthine occurs in Lesch Nyhan disease, an X-linked hereditary disorder caused by deficiency of hypoxanthine-guanine phosphoribosyltranspherase (HPRT) activity (Herderson *et al.*, 1968; Jinnah and Friedmann 2001; Nyhan *et al*., 1965; Rijksen *et al*., 1981). Affected patients present cognitive deficits, hyperuricemia, spasticity, dystonia and self-mutilation behavior, which is characterized by biting of the lips, tongue and fingers with apparent tissue loss (Jinnah and Friedmann, 2001; Jinnah *et al.,* 1990; Matthews *et al.,* 1999; Mizuno, 1986). In addition, studies also show that affected patients present dysfunction of the dopamine transmitter system basal ganglia and a reduction of striatum volume (Jinnah and Friedmann, 2001).

Although underlying mechanisms of brain dysfunction in Lesch Nyhan disease are poorly understood, the accumulation of oxypurines such as hypoxanthine has been proposed to contribute to neurological dysfunction presented (Dasheiff, 1980; Kisch *et al*., 1985; Ma *et al*., 2001; Visser *et al*., 2000). In this context, we have previously demonstrated that hypoxanthine added to the incubation medium inhibits Na^+, K^+ - ATPase activity in plasma synaptic membrane of striatum in six-day-old rats, suggesting a direct effect on the enzyme (Bavaresco *et al.*, 2004). Also, when hypoxanthine was added to striatum homogenate and incubated for 1 hour before plasma synaptic membrane preparation, it reduced Na^+,K^+-ATPase activity, what suggests an indirect action of the oxypurine over enzyme activity. Moreover, hypoxanthine induces oxidative stress in striatum of rats (Bavaresco *et al.,* 2005) and may affect neuronal development by enhancing cell proliferation and impairing morphogenesis (Ma *et al*., 2001). Other

evidence from the literature points that hypoxanthine accumulation provokes dopamine depletion in neuronal cell cultures (Palmour *et al.*,1989).

Interestingly, dopamine (DA) seems to be an important neurotransmitter involved in learning and memory (Gasbarri *et al*., 1996; Myhrer, 2003). DA depletion provoked by 6-hydroxydopamine infusion to rat striatum causes impairment in Water Maze memory (Hagan *et al*., 1983). In addition, some neurodegenerative diseases in which dopaminergic neurons are destroyed offer a clear example of the fundamental role of DA in cognitive abilities (Dubois and Pillon, 1995; Mura and Feldon, 2003). Another amine, serotonin, has also been associated with self aggression (Soderstrom *et al*., 2003).

Considering that (a) Lesch Nyhan patients present cognitive deficits; (b) hypoxanthine accumulation might be toxic to the central nervous system, particularly to striatum; (c) monoamines, such as dopamine and serotonin, play important roles in memory modulation, we decided to investigate the effect of intrastriatal hypoxanthine administration on spatial memory (Morris Water Maze) and in open field tasks. We also studied monoamines levels in rat striatum.

8. Materials and Methods

2.1. Animals and reagents

Sixty-days-old male Wistar rats were obtained from the Central Animal House of the Department of Biochemistry, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil. Animals were maintained on a 12 h light/dark cycle (lights on from 7 a.m. to 7 p.m.) in one air-conditioned constant temperature (22^{o} C) colony room, with free access to a 20% (w/w) protein commercial chow and water. Animal care followed the official governmental guidelines in compliance with the Federation of Brazilian Society

for Experimental Biology and was approved by ethics committee of the Federal University of Rio Grande do Sul, Brazil. All chemicals were purchased from Sigma Chemical Co., St Louis, MO, USA.

2.2. Stereotaxic Surgery and cannula placement

Rats were anesthetized with ketamine and xilazine (75 and 10 mg/kg ip, respectively) and placed in a rodent stereotaxic apparatus. Under stereotaxic guidance, a 27-gauge staineless cannula (0.9 mm O.D.) with an inner needle guide was inserted unilaterally into the right striatum (coordinates relative from bregma: AP, - 0,5 mm; ML - 2,5 mm; V - 2,5 mm from the dura) (Paxinos and Watson, 1986). Two days after the surgery, a 30–gauge needle was inserted into the guide cannula in order to inject buffered hypoxanthine (10 μM) or vehicle (saline) into the right striatum over a 1 min interval.The volume administered (saline or hypoxanthine) was 2μL. Animals were divided into two groups: group 1 (vehicle group), rats that received intrastriatal saline and group 2 (hypoxanthine treated), rats that received intrastriatal hypoxanthine solution (20 pmol/2μL). Hypoxanthine concentration was chosen according to Puig and colleagues (1989). After 7 days of hypoxanthine or saline administration, the animals were subjected to behavioral testing (46 rats). For measurement of monoamine content (20 rats), the rats were sacrificed seven days after drug infusion.

The drug was infused in striatum because patients with this syndrome present characteristic alterations in the basal ganglia (Jinnah and Friedmann, 2001).

2.2. Behavioral procedures

On the 69th day of life, animals were subjected to behavioral testing. We used the Morris water maze, an apparatus widely employed for the study of spatial learning and memory tasks (D'Hooge and De Deyn, 2001; McNamara and Skelton, 1993; Netto *et al*., 1993). The behavioral experiments were conducted between 7h and 12h a.m.

The water maze consisted of a black round tank, 200 cm in diameter and 100 cm high, filled to a depth of 50 cm with water maintained at constant temperature of 23°C. The tank was theoretically divided into four equal quadrants for the purpose of analysis. Several distal visual cues were placed on the walls of the room. Trials were recorded by a video camera mounted above the center of the tank.

a) Reference memory task. The task consisted of 4 training and one test session. In the acquisition phase, rats had daily sessions of 4 trials per day for 6 days to find the platform, submerged 2 cm under the water surface, placed on the center of one of the quadrants of the tank during all training days. For each trial, the rat was placed in water facing tank wall, in one of the 4 starting locations (N, S, W and E). The order of starting position varied in every trial and any given sequence was not repeated on acquisition phase days. Rats were allowed to search for the platform during 60 s and, in the case of failing to find it, they were gently guided to it; all animals were permitted to remain on the platform for 10 s. Latency to find the platform was measured in each trial. The interval between trials was 15-20 min (Netto *et al*., 1993). One day after the last training trial, each rat was subjected to a probe trial in which the platform was removed. We measured four parameters, namely latency to cross on the location of the platform, the number of target crossings and the time spent in target (the quadrant in which the platform was located in the training sessions) and opposite quadrants. These parameters were taken as a measure for spatial memory (Netto *et al*., 1986).

In order to detect motor impairments that could affect performance in experimental groups, the swimming speed was calculated by taking the distance traveled in the first 15 s of the probe trial.

b) Working memory task. After 1 week, the working memory version of Morris water maze was performed. The task consisted of 4 consecutive trials per day, with a 30-second inter-trial interval, when the animals were placed in the tank facing the wall and allowed to search for the submerged platform, positioned on the center of one of the quadrants. Platform position changed every subsequent day during the four testing days. Latencies to find the platform in every first, second, third and fourth trials were calculated considering all testing days so to assess working memory performance (Netto *et al*., 1986).

c) Open field task. The task was run in a wooden box measuring $60 \times 40 \times 50$ cm with a frontal glass wall, whose floor was divided by white lines into 12 equal squares. Animals were placed facing the rear left corner of the arena and observed for 2 min. The number of squares crosses with the four paws from one square to another, the latency in the first square, the number of fecal bolus and rearings was measured as indicative of motor activity (Netto *et al*., 1993).

2.2.6. Determination of striatal monoamines levels

In order to verify the levels of dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), homovallinic acid (HVA), serotonin (5-HT), and 5-hydroxy-indoleacetic acid (5-HIAA), the rats were sacrificed and both striatum were removed and stored at – 70ºC. The endogenous levels of DA, DOPAC, HVA and 5-HIAA were assayed by high performance liquid chromatography with electrochemical detection (HPLC-ED), adapted from Da Cunha and colleagues (2001). The system consisted of a Synergi Fusion-RP C-18 reverse-phase column (150 x 4.6 mm i.d., 4 μm particle size), a LC-10AD pump (Shimadzu), a temperature-controlled oven (30ºC - Shimadzu), and a L-ECD-6A electrochemical detector (Shimadzu). The oxidation potential was fixed at + 0.80 V using a Ag/AgCl working electrode. The tissue samples were homogenized with a vibra-cell ultrasonic processor (Sonics, Newtown, CT, U.S.A.) cell disrupter in 0.1 M perchloric acid. After centrifugation at 15,000 x g for 30 min, 20 μl of the supernatant was injected into the chromatograph. The mobile phase, at a flow rate of 1 ml/min, had the following composition: 15.7 g citric acid, 471.5 ml HPLC-grade water, 78 mg heptanesulfonic acid, 20 ml acetonitrile, 10 ml tetrahydrofuran, pH 3.0. The peak areas of the external standards were used to quantify the sample peaks. The values obtained were expressed as ng/g tissue wet weight.

2.8. Statistical analysis

Differences between groups in the Water Maze procedure were analyzed by repeated measures analysis of variance (ANOVA) and data from the probe trial parameters and the open field test were analyzed by Student's T test. Differences between groups in striatal monoamine levels were analyzed by Student's T test. A $p < 0.05$ was considered significant. Descriptive statistics data were expressed as mean ±

S.E.M. All analyses were performed using the Statistical Package for the Social Science (SPSS) software in a PC- compatible computer.

9. Results

3.1. Experiment 1: Effect of intrastriatal hypoxanthine infusion on reference and working memory task in the Morris water maze.

Hypoxanthine – treated group showed a lower ability to find the platform in the fourth day of training. Two-way ANOVA (days versus groups) revealed a major days effect [$F(1,19) = 4.454$; $p<0.05$] (Fig. 1). Four parameters were evaluated in the test session, latency to cross over the location of the platform, the number of target crossings and the time spent in the target and opposite quadrants (Fig. 2A – D). It is shown that intrastriatal hypoxanthine administration did not affect the time spent in the target quadrant (A) [$t(19)=0.138$; $p>0.05$], in the opposite quadrant (B) [$t(19)=1.245$; $p>0.05$] nor in the number of crossings over the platform area (C) [$t(19)=1.023$; $p>0.05$]; however the latency to cross over the platform location for the first time was significantly decreased when compared to saline group (D) [$t(19)=3.082$; $p <0.05$].

Intrastriatal hypoxanthine administration altered rat performance in the working memory version of Morris Water Maze (Fig 3). Control rats presented significant savings from trial 1 to trial 2 [$t(8)=4.200$; $p<0.05$] and trial 1 to trail 3 [$t(8)=4.483$; $p<0.05$] and hypoxanthine–injected rats showed no significant savings [$t(10)=0.635$ and $t(10)=0.457$, respectively, both $p>0.05$]. There was no difference between groups in trial 1 latency.

No motor deficits were found in rats performing both water maze tasks, as assessed by swim speed; the general mean, considering all experimental groups, was

25.6 cm/s, with $p>0.05$. Additionally, in order to verify motor activity, we submitted the animals to the open field task. Intrastriatal hypoxanthine infusion did not alter any of the four parameters analyzed (numbers of fecal bolli [$t(19)=1.64$; $p>0.05$], rearings [$t(19)=0.316$; $p>0.05$] and crossings [$t(19)=0.799$; $p>0.05$], nor the latency to leave the first square [$t(19)=1.63$; $p>0.05$]) when compared to control group (Table 1).

Experiment 2: Effect of intrastriatal hypoxanthine administration on striatal monoamine content

We analyzed striatal DA, DOPAC, HVA, 5-HT and 5-HIAA content in both experimental groups. Table 2 shows that the administration of hypoxanthine did not cause any modification on DA [$t(17)=1.129$;$p>0.05$], DOPAC [$t(17)=0.790$;$p>0.05$], HVA [$t(17)=0.213$;$p>0.05$] concentration on the striatum of the animals when compared to control group. Additionally, we observed that the contralateral dopaminergic metabolites content was also unaffected in both experimental groups (data not shown). In contrast, the striatal content of 5-HT [$t(17)=2.361$;$p< 0.05$] and 5-HIAA [$t(17)=3.456$;$p<0.05$] were reduced (50%) in hypoxantine-treated rats.

10. Discussion

Lesch Nyhan disease is an X-linked hereditary disorder caused by deficiency of hypoxanthine-guanine phosphoribosyltranspherase activity. Affected patients present hyperuricemia, spasticity, dystonia, mental retardation and self-mutilation behavior

(Jinnah *et al.*, 1990), along with a dysfunction of the dopamine transmitter system of the basal ganglia (Jinnah and Friedmann, 2001). A common feature of Lesch Nyhan is that patients exhibit caracteristically raised level of hypoxanthine in plasma (Harkness *et al*., 1988; Puig and Mateos, 1993; Rosenbloom *et al.,* 1967) urine (Harkness *et al*., 1988) and cerebrospinal fluid (Harkness *et al*., 1988; Puig and Mateos, 1993; Rosenbloom *et al*., 1967). In this context, the accumulation of oxypurines such as hypoxanthine have been proposed to contribute to the neurological dysfunction present in this disease (Dasheiff, 1980; Kish *et al.,* 1985; Ma *et al*., 2001; Visser *et al.,* 2000).

In the present study we demonstrated that intrastriatal injection of hypoxanthine to rats, at the concentration found in Lesch-Nyhan patients, significantly impaired learning/memory in the acquisition phase of the Morris Water Maze and the latency to cross for the first time over the platform location. However, no effect was found in working memory performance. Hypoxanthine-induced deficits cannot be attributed to any decreased in locomotor activity since there wasn't any alterations in the parameters analyzed in the open field task as well as the swim speed (~ 25,6 cm/s) of all groups did not differ.

It has been demonstrated that striatal lesions induced by intrastriatal quinolinic infusion impaired rats performance in Morris Water Maze (Block *et al*., 1993). Additionally, posttraining infusion of D2 dopamine receptor antagonist sulpiride in the posteroventral striatum alters probe trail, indicating an involvement of the striatum in the consolidation of memory (Setlow and McGaugh, 1999).

Although the exact mechanism through which hypoxanthine alters learning/memory in rats is still unknown, evidence from literature indicates some mechanisms that this oxypurine could provoke impairment on memory process. In this context, it has been showed that oxidative stress is associated with memory deficits

(Bickford *et al.,* 1999; Serrano and Klann, 2004; Silva *et al.,* 2004) and that modulation of Na^+, K^+ - ATPase activity is a fundamental mechanism for learning/memory (Brunelli *et al.,* 1997; Sato *et al.,* 2004). Wyse and colleagues (2004) showed that training in inhibitory avoidance tasks inhibited Na^+, K^+ - ATPase activity in rat hippocampus immediately and 6 hours after the training session. On the other hand, we have demonstrated that hypoxanthine inhibits striatal Na^+,K^+-ATPase *in vitro* and *in vivo* (Bavaresco *et al.,* 2004; Bavaresco *et al.,* 2005). Additionally, hypoxanthine induces an increase in reactive oxygen species and/or lipid peroxidation and decreases brain antioxidant capacity (Bavaresco *et al.,* 2005; Beckman *et al.,* 1987).

Considering that the mechanisms that leads to neurobehavioral symptoms in Lesch-Nyhan remains unknown and that a body of evidence have involved some monoamines, such as dopamine and serotonin, in aggressive and self mutilation behavior (Lidberg *et al*., 1984; Soderstrom *et al*., 2003), in the present study we also measured monoamine in striatum of hypoxanthine-treated rats. We used this cerebral structure because patients with Lesch Nyhan disease present characteristic alterations in the basal ganglia (Deutsch *et al*., 2005) and striatum is associated with memory mechanism (Torres *et al*., 2006). Our results showed hypoxanthine-treated rats presented a reduction of striatal 5-HT and 5-HIAA levels, but we did not observed any alteration on dopamine content in these animals. Interestly in agreement with our results, it has been shown that Lesch Nyhan patients may present decreased in 5-TH and 5-HIAA in cerebrospinal fluid (Castells *et al.,* 1979). On the other hand, Geyde (1992) suggests that serotonin and serotonin-gamma aminobutyric acid (GABA) could be used as a treatment for self-injury in this disease since it will increase two endogenous anticonvulsants (serotonin and GABA) and decrease kynurenine and quinolinic acid. However, some studies

demonstrate an increase in 5-HT content in the striatum of Lesch Nyhan patients (Lloyd *et al.,* 1981).

It has also been demonstrated a reduction in urinary secretion of serotonin was seen after oral intake of high doses of hypoxanthine in humans (Manzke and Gustmann, 1989). Evidences in the literature have showed low 5-HIAA concentration in cerebrospinhal fluid in patients with self destructible behavior (Lidberg *et al.,* 1984; Soderstrom *et al.*, 2003). Beside this, an increased HVA: 5-HIAA ratio indicates an alteration in serotoninergic modulation of dopamine activity (Soderstrom *et al.,* 2003). Additionaly, lower concentration of CSF 5-HIAA were associated with impaired memory performance (Bolla *et al.,* 1998).

In conclusion, our results showed that intrastriatal hypoxanthine administration provoked impairment on reference spatial memory in Morris water maze and a reduction of striatum serotonin and its metabolite (5-HIAA) levels without affecting dopamine content, suggesting an alteration of serotoninergic metabolism by hypoxanthine. Considering that Lesch-Nyhan patients present cognitive memory alterations, we can suggest that it might be associated to the accumulation of hypoxanthine.

Acknowledgements

This work was supported in part by grants from CNPq – Brazil, FAPERGS, RS-Brazil, and Programa de Núcleos de Excelência-Financiadora de Estudos e Projetos (PRONEX ll - FINEP-Brazil).

References

Bavaresco, C.S., Zugno, A.I., Tagliari, B., Wannmacher, C.M.D., Wajner, M., and Wyse, A.T.S. (2004). Inhibition of Na^+, K^+ - ATPase activity in rat striatum by metabolites accumulated in Lesch – Nyhan disease. *Int. J. Dev. Neurosci.* **22**:11- 17.

Bavaresco, C.S., Chiarani, F., Matte, C., Wajner, M., Netto, C.A., and Wyse, A.T.S. (2005). Effect of hypoxanthine on Na^+,K^+-ATPase activity and some parameters of oxidative stress in rat striatum. *Brain Res.* **1041**:198-204.

Beckman, J.S., Liu, T.H., Hogan, E.L., Freeman, B.A., and Hsu, C.Y. (1987). Oxygen free radicals and xanthine oxidase in cerebral ischemic injury in the rat. *Soc. Neurosci. Abstr.* **13**:1498.

Bickford, P.C., Shukitt-Hale, B., and Joseph, J. (1999). Effects of aging on cerebellar noradrenergic function and motor learning: nutritional interventions. *Mech. Ageing Dev.* **111**:141-154.

Block, F., Kunkel, M., and Schwarz, M. (1993). Quinolinic acid lesion of the striatum induces impairment in spatial learning and motor performance in rats. *Neurosci. Lett.* **149**:126 -128.

Bolla, K.I., McCann, U.D., and Ricaurte, G.A. (1998). Memory impairament in abstinent MDMA ("Ecstasy") users. *Neurology* **51**: 1529-1530.

Brunelli, M., Garcia-Gil, M., Mozzachiodi, R., Scuri, R., and Zaccardi, M.L. (1997). Neurobiological principles of learning and memory. *Arch. Ital. Biol.* **135:**15-36.

Carbon, M., and Marie, R.M. (2003). Functional imaging of cognition in Parkinson's disease. *Curr. Opin. Neurol.* **16:** 475-480.

Castells, S., Chakrabarti, C., Winsberg, B.G., Hurwic, M., Perel, J.M., and Nyhan, W.L. (1979). Effects of L-5-hydroxytryptophan on monoamine and amino acids turnover in the Lesch Nyhan syndrome. *J. Autism. Dev. Disord.* **9**: 95-103.

Da Cunha, C., Gevaerd, M.S., Vital, M.A.B.F., Myioshi, E., Andreatini, R., Silveira, R, Takahashi, R,N., and Canteras, N.S. (2001). Memory in rats with nigral lesion induced by MTPT: A model for early Parkinson's disease amnesia. *Behav. Brain Res.* **124**:9 – 18.

Dasheiff, R.M. (1980). Benzodiazepinic treatment for Lesch-Nyhan syndrome? *Dev. Med. Child. Neurol.* **22**:101-102.

Deutsch, S.I., Long, K.D., Rosse, R.B., Mastropaolo, J., and Eller, J. (2005). Hypothesized deficiency of guanine-based purines may contribute to abnormalities of neurodevelopment, neuromodulation and neurotransmission in Lesch – Nyhan disease. *Clin. Neuropharmacol.* **28**:28 – 37.

D´Hooge, R., and De Deyn, P.P. (2001). Application of the Morris water maze in the study of learning and memory. Brain Res. Brain Res. Rev. **36**:60 – 90.

Dubois, B., and Pillon, B. (1995). Do cognitive changes of Parkinson´s disease result from dopamine depletion? J. Neural Transm. **45**:27 – 34.

Gasbarri, A., Sulli, A., Innocenzi, R., Pacitti, C., Brioni, J.D. (1996). Spatial memory impairment induced by lesion of the mesohipocampal dopaminergic system in the rat. *Neurosci.* **74**:1037-1044.

Geyde, A. (1992). Serotonin-GABA treatment is hypothesized for self-injury in Lesch Nyhan syndrome. *Med. Hypotheses* **38**: 325-328.

Hagan, J.J., Alpert, J.E., Morris, R.G., and Iversen, S.D. (1983). The effects of central cathecolamine depletions on spatial learning in rats. *Behav. Brain Res.* **9**:83 – 104.

Harkness, R.A., McCreanor, G.M., and Watts, R.W. (1988). Lesch-Nyhan syndrome and its pathogenesis: purine concentrations in plasma and in urine with metabolite profiles in CSF. *J. Inher. Metab. Dis.* **11**:239 - 252.

Herderson, J.F. (1968). Possible functions of hypoxanthine – guanine phosphoribosyltransferase and their relation to the biochemical pathology of the Lesch-Nyhan syndrome. *Fed. Proc*. **2**:302 - 304.

Jinnah, H.A., Gage, F.H., and Friedmann, T. (1990). Animal models of Lesch – Nyhan syndrome. *Brain Res. Bull.* **25**:467 – 75.

Jinnah, H.A., and Friedmann, T. (2001). Lesch Nyhan disease and it variants. In (C.R. Scriver, A.L. Beaudet, W.S. Sly, D. Valle, eds), *The Metabolic and Molecular Basis of Inherited Disease*, 8th end*.,* McGraw-Hill, New York, pp. 2537-2569.

Kisch, S.J., Fox, I.H., Kapur, B.M., Lloyd, K.G., and Hornykiewicz, O. (1985). Brain benzodiazepine receptor binding and purine concentration in Lesch-Nyhan syndrome. *Brain Res*. **336**:117 - 23.

Lidberg, L., Asberg, M., and Sundqvist-Stensman, U.B. (1984). 5-hydroxyindoliacetic acid levels in attempted suicides who have killed their children. *Lancet* **2**(8408): 928.

Lloyd, K.G, Hornykiewicz, O., Davidson, L., Shannak, K., Farley, I., Goldstein, M., Shibuya, M., Kelley, W.N., and Fox, I.H. (1981). Biochemical evidence of dysfunction of brain neurotransmitter in Lesch Nyhan syndrome. *N. Engl. J. Med*. **305**:1106-1111.

Ma, M.H.Y., Stacey, N.C., and Connolly, G.P. (2001). Hypoxanthine impairs morphogenesis and enhances proliferation of a neuroblastoma model of Lesch Nyhan syndrome. *J. Neurosci. Res.* **63**:500 - 8.

Manzke, H., and Gustmann, H. (1989). Reduced urinary serotonin excretion after intake of high doses of hypoxanthine. *Eur .J. Pediatr.* **148**: 337-340.

Matthews, W.S., Solan, A., Barabas, G., and Robey, K. (1999). Cognitive functioning in Lesch-Nyhan syndrome: a 4-year follow-up study. *Dev. Med. Child. Neurol.* **41**:260-262.

Mc Namara, R.K., and Skelton, R.W. (1993). The neuropharmacological and neurochemical basis of place leraning in the Morris water maze. *Brain Res. Brain Res. Rev.* **18**:33 – 49.

Mizuno, T. (1986). Long-term follow-up of ten patients with Lesch-Nyhan syndrome. *Neuropediatrics* **17**:158 – 161.

Mura, A., and Feldon, J. (2003). Spatial learning in rats is impaired after degeneration of the nigrostriatal dopaminergic system. *Mov. Disord*. **18**:860 – 871.

Myhrer, T. (2000). Neurotransmitter systems involved in learning and memory in the rat: a meta-analysis based on studies of four behavioral tasks. *Brain Res. Rev.* **41:**268 –287.

Netto, C.A., Hodges, H., Sinden, J.D., Le Peilet, E., Kershaw, T., Sowinski, P., Meldrum, B.S.and Gray, J.A. (1993). Effects of fetal hippocampal field grafts on ischaemic-induced deficits in spatial navigation in the water maze. *Neuroscience* **54**:69 – 92.

Netto, C.A., Dias, R.D., and Izquierdo, I. (1986). Differential effect of posttraining naxolane,beta-endorphin, leu-enkephalin and electro-convulsive shock administration upon memory of an open field habituation and of a water-finding task. *Psychoneuroendocrinology* **11**:437 –4 46.

Nyhan, W.L., and Oliver, W.J., and Lesch, M. (1965). A familial disorder or uric acid metabolism and central nervous system function II. J. Pediatr. **67**:439 -444.

Palmour, R.M., Heshka, T.W., and Ervin, F.R. (1989). Hypoxanthine accumulation and dopamine depletion in Lesch-Nyhan disease. *Adv. Exp. Med. Biol.* **253**:165 - 72.

Paxinos, G., and Watson, C. (1986). *The rat brain in stereotaxic coordinates*. 2nd ed. San Diego: Academic Press.

Puig, J.G., Mateos, F.A., Jimenez, M.L., Ramos, T., Capitan, M.C., and Gil, A.A. (1989). Impaired renal excretion of hypoxanthine and xanthine in primary gout. Adv. Exp. Med. Biol. **253A:** 269-76.

Puig, J.G., and Mateos, F.A. (1993). *The biochemical basis of HGPRT deficiency.* In: (U. Gresser, ed). Molecular genetics, biochemistry and clinical aspects of inherited disorders of purine and pyrimidine metabolism. Springer-Verlag, New York. pp. 12-26.

Rijksen, G., Staal, G.E.J., Van der Vlist, M.J.M., Beerner, F.A., Troost, J., Gutensohn, W., van Laarhoven, J.P., and de Bruyn, C.H. (1981).Partial hypoxanthine-guanine phosphoribosyl transferase deficiency with full expression of the Lesch – Nyhan syndrome. *Hum. Gen.* **57**:39 - 47.

Rosenbloom, R.M., Henderson, J.F., Caldwell, I.C., Kelley, W.N., and Seegmiller, J.E. (1967). Inherited disorder of purine metabolism. *JAMA* **202**:175 -77.

Sato, T., Tanaka, K., Ohnishi, Y., Teramoto, T., Irifune, M., and Nishikawa, T. (2004). Effects of steroid hormones on (Na^+, K^+)-ATPase activity inhibition-induced amnesia on the step-through passive avoidance task in gonadectomized mice. *Pharmacol. Res.* **49**:151-159.

Serrano, F., and Klann, E. (2004). Reactive oxygen species and synaptic plasticity in the aging hippocampus. *Ageing Res. Rev.* **3**:431- 43.

Setlow, B., and McGaugh, J.L. (1999). Involvement of the posteroventral caudate-putamen in memory consolidation in the Morris water maze. Neurobiol. Learn. Mem. **71**:240- 247.

da Silva, A.L., Piato, A.L., Bardini, S., Netto, C.A., Nunes, D.S., and Elisabetsky E. (2004). Memory retrieval improvement by Ptychopetalum olacoides in young and aging mice. J. Ethnopharmacol. 95(2-3):199-203.

Soderstrom, H., Blennow, K., Sjodin, A.K., and Forsman, A. (2003). New evidence for an association between the CSF HVA: 5-HIAA ratio and psychopathic traits. J. Neurosurg. Psychiatric. **74**: 918-921.

Torres, J.B., Assunção, J., Farias, J.A., Kahwage, R., Lins, N., Passos, A., Quintairos, A., Trevia, N., and Diniz, C.W. (2006). NADPH-diaphorase histochemical changes in the hippocampus, cerebellum and striatum are correlated with different modalities of exercise and watermaze performances. Exp Brain Res., in press.

Visser, J.E., Bär, P.R., and Jinnah HA. (2000). Lesch-Nyhan disease and the basal ganglia. *Brain Res. Bull.* **32**:449 -475.

Wyse, A.T.S., Bavaresco, C.S., Reis, E.A., Zugno, A.I., Tagliari, B., Calcagnotto, T., and Netto, C.A. (2004). Training in inhibitory avoidance causes a reduction of Na^+, K^+ - ATPase activity in rat hippocampus. Physiol. Behav. **80**:475 – 479.

Figure 1. Effect of intrastriatal hypoxanthine injection on performance of spatial memory acquisition phase. Data are expressed as means ± SEM for 9-11 animals in each group. *p< 0.05 compared to sham group on the 4th day of training session. (ANOVA). Hpx – hypoxanthine

Figure 2. Effect of intrastriatal hypoxanthine injection on performance of spatial memory in test session parameters, namely time spent in the target quadrant (A), time spent in opposite quadrant (B), latency to first cross over the platform location (C) and the number of crossings over platform location (D). Data are means ± SEM for 9-11 animals in each group. *p< 0.01 compared to control(ANOVA). Hpx - hypoxanthine

Figure 3. Effect of intrastriatal hypoxanthine injection on performance in working memory version of Morris Water Maze spatial task. Data are expressed as means ± SEM for 9-11 animals in each group. * Significant difference between T1-T2 and T1-T3, p>0.05. Hpx – hypoxanthine.

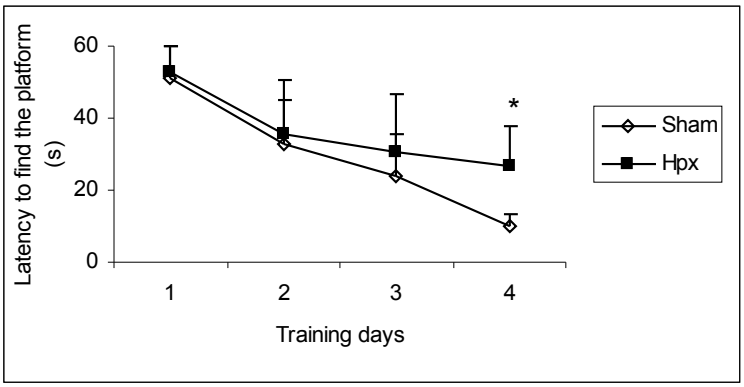

Latency to find the platform (s)
60
40
20
0
1
2
3
4
Training days
*
Sham
Hpx

Figure 1

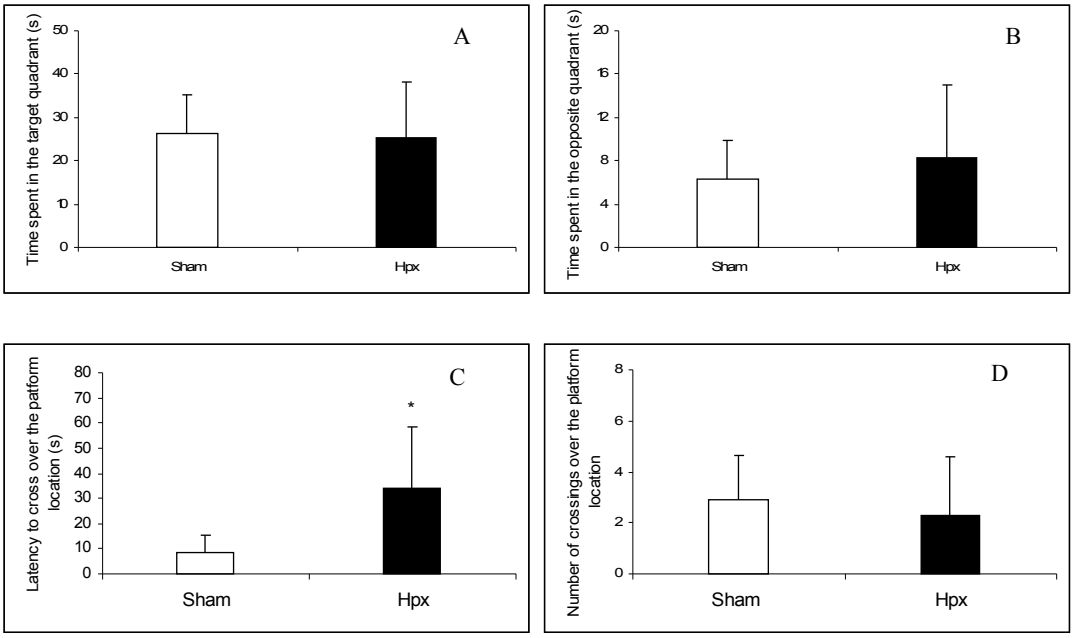

A
Time spent in the target quadrant (s)
50
40
30
20
10
0
Sham
Hpx
B
Time spent in the opposite quadrant (s)
20
16
12
8
4
0
Sham
Hpx
C
Latency to cross over the patform location (s)
80
70
60
50
40
30
20
10
0
*
Sham
Hpx
D
Number of crossings over the platform location
8
6
4
2
0
Sham
Hpx

Figure 2

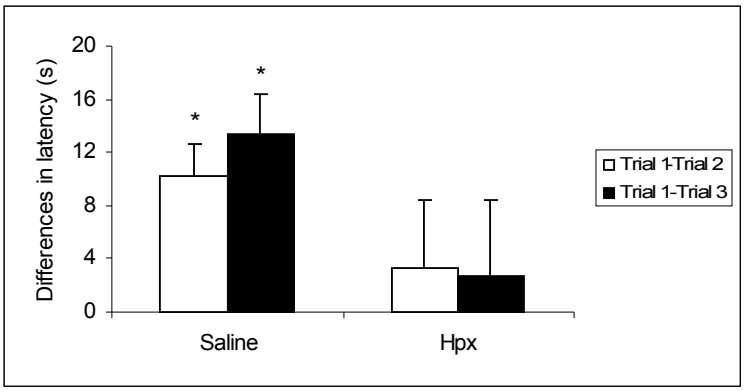

Differences in latency (s)
20
16
12
8
4
0
*
*
Saline
Hpx
Trial 1-Trial 2
Trial 1-Trial 3

Figure 3

Table 1. Effect of intrastriatal hypoxanthine administration on performance (number of crossings, rearings and fecal bolus and the latency in the first square) in the open field task.

	Groups	
Open Field	Sham	Hypoxanthine
Number of crossings	37.25 ± 12.35	33.44 ± 8.18
Number of fecal bolus	3.41 ± 1.88	2.00 ± 2.06
Number of rearings	9.91 ± 6.12	9.22 ± 2.68
Latency in the first square	2.50 ± 3.12	4.88 ± 3.58

Data are presented as means ± SD for 9 – 12 animals in each group.

There was no significant difference between groups, $p > 0.05$. (Student's T test) Hpx– hypoxanthine.

Capítulo VI – Artigo 06

Intrastriatal injection of hypoxanthine impairs memory formation on step-down inhibitory avoidance task in rats.

Caren Serra Bavaresco, Juliana Ben, Fabria Chiarani, Carlos Alexandre Netto and Angela Terezinha de Souza Wyse[1]

Aceito para publicação na revista Pharmacology, Biochemical and Behaviour, 2008.

Intrastriatal injection of hypoxanthine impairs memory formation of step-down inhibitory avoidance task in rats

Caren Serra Bavaresco, Juliana Ben, Fabria Chiarani, Carlos Alexandre Netto and Angela Terezinha de Souza Wyse[1]

Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil;

[1]Address reprint request to: Dr. Angela T.S. Wyse, Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2600-Anexo, CEP 90035-003 Porto Alegre, RS, Brazil, Telephone number: 55 51 33085573; E-mail: wyse@ufrgs.br.

Abstract

The aim of this study was to investigate the effects of intrastriatal injection of hypoxanthine, the major compound accumulated in Lesch-Nyhan disease, on performance step-down inhibitory avoidance task in the rat. Male adult Wistar rats were divided in two groups: (1) saline-injected and (2) hypoxanthine-injected group. Treated-group received intrastriatal hypoxanthine solution 30 min before training session (memory acquisition) or immediately after training session (memory consolidation) or 30 before test session (memory retrieval) on step-down inhibitory avoidance task. Results show that hypoxanthine administration caused significant memory impairment in all periods tested. These results show that intrastriatal hypoxanthine administration provoked memory process impairment of step-down inhibitory avoidance task, an effect that might be related to the cognitive memory alterations in Lesch-Nyhan patients.

Key Words: Lesch-Nyhan disease; Intrastriatal hypoxanthine administration; memory; step-down inhibitory avoidance task

1. Introduction

Tissue accumulation of hypoxanthine occurs in patients with Lesch Nyhan disease, an X-linked hereditary disorder caused by deficiency of hypoxanthine-guanine phosphoribosyltranspherase (HPRT) activity (Nyhan et al., 1965). Affected patients present cognitive deficits, hyperuricemia, spasticity, dystonia and self-mutilation behavior characterized by biting of the lips, tongue and fingers with apparent tissue loss (Jinnah and Friedmann, 2001; Matthews, 1999). In addition, studies also show that affected patients present dysfunction of dopamine transmitter system in basal ganglia and a reduction of striatum volume (Jinnah and Friedmann, 2001, Palmour et al., 1989).

Accumulation of hypoxanthine has been proposed to contribute to neurological dysfunction presented in patients with Lesch Nyhan disease [Brunori, 2001; Kisch et al., 1985; Ma et al., 2001; Visser et al., 2000). In this context, Bavaresco and colleagues (2007a) demonstrated that intrastriatal injection of hypoxanthine in rats, at the concentration found in Lesch-Nyhan patients, significantly impaired spatial learning/memory in the acquisition phase of the Morris Water Maze and decreased striatal levels of serotonin (5-HT) and 5- hydroxy-indoleacetic acid (5-HIAA). Beside this, Ägren and colleagues (1983) indicated correlations between higher levels of hypoxanthine in cerebrospinal fluid (CSF) and memory disturbance.

We also showed a decrease on Na^+, K^+ - ATPase activity and total radical-trapping antioxidant parameter (TRAP) in striatum, hippocampus and cerebral cortex of rats, as well as an increase in chemiluminescence, in the same cerebral structures, 30 min after hypoxanthine infusion in rat striatum (Bavaresco et al., 2007b) In addition, studies show that hypoxanthine may affect neuronal development by enhancing cell proliferation and impairing morphogenesis (Ma et al., 2001).

It has been shown that hypoxanthine infusion impairs memory in task of water Maze in rats (Bavaresco et al., 2007a) in which declarative or spatial component of a task can be evaluated. Other tasks, like step-down inhibitory avoidance, evaluate a conditioned avoidance response task, are also important to evaluate memory/learning (Rossato et al., 2006) (Bavaresco et al., 2007a). Evidence from literature pointed that dorsal striatum is involved in various types of learning/memory such as procedural learning, reward-association and emotional learning (Boussaoud and Kermadi, 1997; Ragozzino et al.,2001; Gill and Mizumori, 2006; Ferreira et al., 2008). In this context, Packard and colleagues (2006) showed that post-training infusion of metabotropic glutamate receptor (mGluR) antagonist in dorsal striatum impaired retention on step-down inhibitory avoidance task. Report from literature indicates that lesions to central structures, including striatum and hippocampus could affect the acquisition, consolidation and retrieval memory phases indicating behavioral differences between memory processes in the first few hours or in the following few days, which suggest participation of different mechanisms (Medina et al., 1999). Moreover, it has been proposed that Na^+, K^+ - ATPase activity inhibition (Wyse et al., 2004) and oxidative stress induction (Delwing et al.2006; Reis et al., 2002) could impair memory formation in rats.

In the present study we investigated the effect of intrastriatal hypoxanthine infusion on step-down inhibitory avoidance task at different periods. The drug was infused into the striatum because patients with this syndrome present characteristic alterations in the basal ganglia (Jinnah and Friedmann, 2001).

2. Materials and Methods

2.1. Animals and reagents

Sixty-days-old male Wistar rats were obtained from the Central Animal House of the Department of Biochemistry, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil. Animals were maintained on a 12 h light/dark cycle (lights on from 7 a.m. to 7 p.m.) in air-conditioned constant temperature (22^{o} C) colony room, with free access to a 20% (w/w) protein commercial chow and water. Animal care followed the official governmental guidelines in compliance with the Federation of Brazilian Society for Experimental Biology and was approved by Ethics Committee of the Federal University of Rio Grande do Sul, Brazil. All chemicals were purchased from Sigma Chemical Co., St Louis, MO, USA.

2.2. Stereotaxic surgery and cannula placement

Rats were anesthetized with ketamine and xilazine (75 and 10 mg/kg ip, respectively) and placed in a rodent stereotaxic apparatus. Under stereotaxic guidance, a 27-gauge staineless cannula (0.9 mm O.D.) with an inner needle guide was inserted unilaterally into the right striatum (coordinates relative from bregma: AP, - 0,5 mm; ML - 2,5 mm; V - 2,5 mm from the dura) (Paxinos and Watson, 1986). In our experiments, we utilized a single cannula implanted into the striatum as described by Sánchez-Iglesias (2007). Two days after the surgery, a 30–gauge needle was inserted into the guide cannula in order to inject buffered hypoxanthine (10 μM) or vehicle (saline) into the right striatum over a 1 min interval. The volume administered (saline or hypoxanthine) was 2 μL. Animals were divided into two groups: group 1 (vehicle group), rats that received intrastriatal saline and group 2 (hypoxanthine-treated), rats that received intrastriatal hypoxanthine solution (20pmol/2μL). Hypoxanthine concentration was

chosen according to Puig and colleagues (1989).

2.3. Drug administration procedure

In order to evaluate the effect of hypoxanthine on memory processing phases (acquisition, consolidation and retrieval), drugs were infused into the right striatum at different periods: 30 min before training session (memory acquisition), immediately after training session (memory consolidation) and 30 min before test session (memory retrieval).

2.4. Behavioral procedures

2.4.1. Step-down inhibitory avoidance task

On the 63rd day of life, animals were subjected to behavioral testing. We used the step-down inhibitory avoidance task since it has been widely used in the study of memory formation (Izquierdo and Medina, 1997; Prado-Alcalá et al., 2003; Wyse et al., 2004); behavioral experiments were conducted between 11 h a.m. and 15 h p.m.

Animals were subjected to training and test sessions in a step-down inhibitory avoidance task with an interval of 24h in between (Izquierdo and Medina, 1997). This task involves learning not to step down from a platform in order to avoid a mild foot shock (Izquierdo and Medina, 1997). The task was carried out in an automatically operated, brightly illuminated box. The left extreme of the grid was covered by a 7.0 cm wide, 2.5 cm high formic platform. Animals were placed on the platform and their latency to step down, placing their four paws on the grid (42.0 X 25.0 cm grid of parallel 0.1 cm caliber stainless steel bars spaced 1.0 cm apart), was measured. In test sessions, no foot shock was delivered and step-down latency (with a ceiling of 180 s) was used as

a measure of memory retention, as described in previous reports (Izquierdo and Medina, 1997; Reis-Lunardelli et al., 2007).

2.5. Statistical analysis

Differences between test and training latency differences on inhibitory avoidance task were assessed by individual (two tailed) Mann-Whitney U tests, $p<0.05$ was considered significant. Descriptive statistics data were expressed as median (interval interquartile). All analyses were performed using the Statistical Package for the Social Science (SPSS) software in a PC- compatible computer.

3. Results

3.1. Experiment 1: Effect of intrastriatal hypoxanthine infusion 30 min before training session on step-down inhibitory avoidance task.

Fig. 1 shows the effect of intrastriatal hypoxanthine infusion 30 min before training on step-down inhibitory avoidance task. Latency differences in training were not significant among control and hypoxanthine groups in Mann-Whitney U test (U= 45.50, $p>0.05$). Latency differences in test performance were significant among control and hypoxanthine groups according to Mann-Whitney U test (U= 18.00, $p<0.05$).

3.2. Experiment 2: Effect of intrastriatal hypoxanthine infusion immediately after training session on step-down inhibitory avoidance task.

Fig. 2 shows the effect of intrastriatal hypoxanthine infusion immediately after step-down inhibitory avoidance training. Latency differences in training were not significant among control and hypoxanthine groups (U= 64.00, $p>0.05$), however there

was a latency differences in test performance were significant among control and hypoxanthine groups according to Mann-Whitney U test (U= 26.00, $p<0.05$).

3.3. Experiment 3: Effect of intrastriatal hypoxanthine infusion 30 min before test session on step-down inhibitory avoidance task.

Fig. 3 illustrates the effect of intrastriatal hypoxanthine infusion 30 min before test session on step-down inhibitory avoidance task. Latency differences in training were not significant (U= 23.00, p>0.05), however, there was a latency differences in test performance were significant among control and hypoxanthine groups according to Mann-Whitney U test (U= 15.00, $p<0.05$).

4. Discussion

In the present study we investigated the effect of intrastriatal hypoxanthine administration on step-down inhibitory avoidance task. Our results demonstrate that hypoxanthine infusion, at the concentration found in Lesch-Nyhan patients, significantly impaired learning/memory 30 min before training or test session as well as immediately after training. This is in agreement with a previous work which showed a significant impairment on learning/memory on the Morris Water Maze task (Bavaresco et al., 2007a). The results obtained in our study probably are not attributed to motor deficits, since we have previously demonstrated that hypoxanthine administration did not alter the open field task (Bavaresco et al., 2007a).

Although the exact mechanism through which hypoxanthine alters learning/memory in rats is still unknown, it has been showed that modulation of Na^+, K^+ - ATPase activity is a fundamental mechanism for learning/memory Brunelli et al., 1997; Reis-Lunardelli

et al., 2007; Sato et al., 2004), for long-term potentiation (LTP) induction (Glushchenko and Izvarina, 1997) and learning in distinct models (Brunori, 2001). For instance, bilateral infusion of ouabain, a specific inhibitor of Na^+, K^+ - ATPase activity, on chick forebrain causes inhibition on consolidation phase with retention loss persisting at least 24 hours after training (Gibbs et al., 2003, Sherry and Crowe, 2007) and another study showed Na^+, K^+ - ATPase inhibition in rat hippocampus immediately and 6 hours after training session on inhibitory avoidance task (Wyse et al., 2004).

It has been shown that 30 min after intrastriatal hypoxanthine infusion, Na^+,K^+-ATPase activity in striatum, hippocampus and cerebral cortex of rats was significantly decreased (Bavaresco et al., 2007b). Moreover, hypoxanthine *in vitro* significantly inhibits Na^+,K^+-ATPase activity from purified synaptic plasma membrane, suggesting a direct action of these compounds on the enzyme (Bavaresco et al., 2004). It is then conceivable that the inhibitory effect elicited by hypoxanthine on Na^+,K^+-ATPase activity could be one of the mechanism involved on the memory impairment observed.

The iinduction of oxidative stress caused by hypoxanthine infusion should not be excluded, since oxidative stress is also associated with memory deficits (Bickford et al., 1999; Serrano and Klan, 2004). Evidence showed that hypoxantine induces an increase in reactive oxygen species and/or lipid peroxidation and decreases brain antioxidant capacity (Bavaresco et al., 2005, Bavaresco et al., 2006, Bavaresco et al., 2007b, Beckman et al., 1987). It has been shown that the formation of free radicals by hypoxanthine/xanthine oxidase could contribute to the destruction of blood-brain barrier observed in ischemic brain tissue (Beckman et al., 1987). Moreover, oxidative stress induced by hypoxanthine inhibited Na^+,K^+-ATPase activity in striatum, cerebral cortex and hippocampus of rats (Bavaresco et al., 2004). In fact, it is then possible to suggest

that the imbalance between free radical production and antioxidant defenses caused by hypoxanthine administration could lead to memory deficits found in the present study.

The biochemical events involved in memory process could be also modulated by neurotransmitters like serotonin and GABA. In this context, Prado-Alcalá and colleagues (2003) showed that post-training administration of the 5-HT2 receptor blocker ketanserine produced a memory retention deficit in rats. In addition, Ticku and Burch (1980) demonstrated that hypoxanthine could inhibit benzodiazepine and GABA binding to its receptor-like sites in rat brain membrane. Also elevate extracellular levels of hypoxanthine could bind to benzodiazepine agonist sites in GABA(A) receptor inhibiting memory process (Deutsch et al., 2005, Izquierdo and Medina, 1997, Savic et al., 2005). These evidences give support to the experimental impairment on memory formation obtained in present study.

In conclusion, our results show that intrastriatal hypoxanthine administration provoked memory impairment in rats submitted to step-down inhibitory avoidance task. Considering that Lesch-Nyhan patients present cognitive memory alterations, we suggest that it might be associated to the accumulation of hypoxanthine in brain.

Acknowledgements

This work was supported in part by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq – Brazil) and by the FINEP Research Grant “Rede Instituto Brasileiro de Neurociência (IBN-Net) - # 01.06.0842-00”.

References

Ägren H, Niklasson F, Hallgren R. Brain purinergic activity linked with depressive symptomatology: hypoxanthine and xanthine in CSF of patients with major depressive disorders. Psychiatry Res 1983; 9: 179-89.

Bavaresco CS, Zugno AI, Tagliari B, Wannmacher CMD, Wajner M, Wyse ATS. Inhibition of Na^+, K^+ - ATPase activity in rat striatum by metabolites accumulated in Lesch – Nyhan disease. Int. J. Dev. Neurosci 2004; 22: 11- 7.

Bavaresco CS, Chiarani F, Matte C, Wajner M, Netto CA, Wyse ATS, Effect of hypoxanthine on Na^+,K^+-ATPase activity and some parameters of oxidative stress in rat striatum. Brain Res 2005; 1041: 198-204.

Bavaresco CS, Chiarani F, Wajner M, Netto CA, Wyse ATS. Intrastriatal hypoxanthine administration affects Na^+,K^+-ATPase, acetylcholinesterase and catalase activities in striatum, hippocampus and cerebral cortex of rats. Int J Dev Neurosci 2006; 24: 411-17.

Bavaresco CS, Chiarani F, Duringon E, Ferro MM, Cunha CD, Netto CA, et al. Intrastriatal injection of hypoxanthine reduces striatal serotonin content and impairs spatial memory performance in rats. Metab Brain Dis 2007a; 22: 67-76.

Bavaresco CS, Chiarani F, Wannmacher CM, Netto CA, Wyse ATS. Intrastriatal hypoxanthine reduces Na($^+$),K ($^+$)-ATPase activity and induces oxidative stress in the rats. Metab Brain Dis 2007b; 22: 1-11.

Beckman JS, Liu TH, Hogan EL, Freeman BA, Hsu CY. Oxygen free radicals and xanthine oxidase in cerebral ischemic injury in the rat. Soc Neurosci Abstr 1987; 13: 1498.

Bickford PC, Shukitt-Hale B, Joseph J. Effects of aging on cerebellar noradrenergic function and motor learning: nutritional interventions, Mech Ageing Dev 1999; 111: 141-54.

Boussaoud D, Kermadi I. The primate striatum: neuronal activity in relation to spatial attention versus motor preparation. Eur J Neurosci. 1997; 10:2152-2168.

Brunelli M, Garcia-Gil M, Mozzachiodi R, Scuri R, Zaccardi ML. Neurobiological principles of learning and memory. Arch Ital Biol 1997; 135: 15-36.

Brunori M. Nitric oxide, cytochrome-*c* oxidase and myoglobin. Trends Biochem Sci 2001; 26: 21–3.

Delwing D, Bavaresco CS, Monteiro SC, Matte C, Netto CA, Wyse AT. Alpha-Tocopherol and ascorbic acid prevent memory deficits provoked by chronic hyperprolinemia in rats. Behav Brain Res 2006; 168**:** 185-9.

Deutsch SI, Long KD, Rosse RB, Mastropaolo J, Eller J. Hypothesized deficiency of guanine-based purines may contribute to abnormalities of neurodevelopment, neuromodulation and neurotransmission in Lesch – Nyhan disease. Clin Neuropharmacol 2005; 28: 28 – 37.

Devan BD, White NM. Parallel information processing in the dorsal striatum: relation to hippocampal function. J Neurosci 1999; 19**:** 2789-98.

Ferreira TL, Shammah-Lagnado SJ, Bueno OF, Moreira KM, Fornari RV, Oliveira MG. The indirect amygdala-dorsal striatum pathway mediates conditioned freezing: Insights on emotional memory networks. Neuroscience. 2008; 153: 84-94.

Gibbs ME, Andrew RJ, Ng KT. Hemispheric lateralization of memory stages for discriminated avoidance learning in the chick. Behav Brain Res 2003; 139: 157-65.

Gill KM, Mizumori SJ Context-dependent modulation by D(1) receptors: differential effects in hippocampus and striatum. Behav Neurosci. 2006; 120: 377–392.

Glushchenko TS, Izvarina NL. Na^{+},$K(^{+})$-ATPase activity in neurons and glial cells of the olfactory cortex of the rat brain during the development of long-term potentiation. Neurosci Behav Physiol 1997; 27: 49-52.

Izquierdo I, Medina JH. Memory formation: the sequence of biochemical events in the hippocampus and its connection to activity in other brain structures, Neurobiol Learn Mem 1997; 68: 285-316. Review.

Jinnah HA, Friedmann T. Lesch Nyhan disease and it variants. In Scriver CR, Beaudet AL, Sly WS, Valle D, editors. The Metabolic and Molecular Basis of Inherited Disease. New York: McGraw-Hill; 2001. p. 2537-69.

Kesner RP, Rogers J. An analysis of independence and interactions of brain substrates that subserve multiple attributes, memory systems, and underlying processes. Neurobiol Learn Mem 2004; 82: 199-215. Review.

Kisch SJ, Fox IH, Kapur BM, Lloyd KG, Hornykiewicz O. Brain benzodiazepine receptor binding and purine concentration in Lesch-Nyhan syndrome. Brain Res 1985; 336: 117 - 23.

Ma MHY, Stacey NC, Connolly GP. Hypoxanthine impairs morphogenesis and enhances proliferation of a neuroblastoma model of Lesch Nyhan syndrome. J Neurosci Res. 2001; 63: 500 -08.

Matthews WS, Solan A, Barabas G, Robey K. Cognitive functioning in Lesch-Nyhan syndrome: a 4-year follow-up study. Dev Med Child. Neurol 1999; 41: 260-2.

Medina JH, Schroder N, Izquierdo I. Two different properties of short- and long-term memory. Behav Brain Res 1999; 103; 119-21.

Nyhan WL, Oliver WJ, Lesch M. A familial disorder or uric acid metabolism and central nervous system function II. J Pediatr 1965: 67: 439 -44.

Packard MG, Vecchioli SF, Schroeder JP, Gasbarri A. Task-dependent role for dorsal striatum metabotropic glutamate receptors in memory. Learn Mem. 2001; 8: 96-103.

Palmour RM, Heshka TW, Ervin FR. Hypoxanthine accumulation and dopamine depletion in Lesch-Nyhan disease. Adv Exp Med Biol 1989; 253: 165 - 72.

Paxinos G, Watson C. The rat brain in stereotaxic coordinates. 2rd ed. San Diego: Academic Press; 1986.

Prado-Alcalá RA, Solana-Figueroa R, Galindo LE, Medina AC, Quirarte GL. Blockade of striatal 5-HT2 receptors produces retrograde amnesia in rats. Life Sci 2003; 74: 481-88.

Puig JG, Mateos FA, Jimenez ML, Ramos T, Capitan MC, Gil AA. Impaired renal excretion of hypoxanthine and xanthine in primary gout. Adv Exp Med Biol 1989; 253A: 269-76.

Ragozzino KE, Leutgeb S, Mizumori SJ. Dorsal striatal head direction and hippocampal place representations during spatial navigation. Exp Brain Res. 2001; 139: 372-376.

Reis EA, Zugno AI, Franzon R, Tagliari B, Matte C, Lammers ML, et al. Pretreatment with vitamins E and C prevent the impairment of memory caused by homocysteine administration in rats. Metab Brain Dis 2002; 17: 211-17.

Reis-Lunardelli EA, Castro CC, Bavaresco C, Coitinho AS, da Trindade LS, Perrenoud MF, et al. Effects of thyroid hormones on memory and on $Na(^{+})$, $K(^{+})$-ATPase activity in rat brain. Curr Neurovasc Res 2007; 4: 184-93.

Rossato JI, Zinn CG, Furini C, Bevilaqua LR, Medina JH, Cammarota M, et al. A link between the hippocampal and the striatal memory systems of the brain. An Acad Bras Cienc 2006; 78: 515-23. Review.

Sánchez-Iglesias S, Rey P, Méndez-Alvarez E, Labandeira-García JL, Soto-Otero R. Time-course of brain oxidative damage caused by intrastriatal administration of 6-hydroxydopamine in a rat model of Parkinson's disease. Neurochem Res 2007; 32 :99-105.

Sato T, Tanaka K, Ohnishi Y, Teramoto T, Irifune M, Nishikawa T. Effects of steroid hormones on (Na^+, K^+)-ATPase activity inhibition-induced amnesia on the step-through passive avoidance task in gonadectomized mice. Pharmacol Res 2004; 49: 151-59.

Savic MM, Obradovic DI, Ugresic ND, Bokonjic DR. Memory effects of benzodiazepines: memory stages and types versus binding-site subtypes. Neural Plast 2005; 12: 289-98. Review.

Serrano F, Klann E. Reactive oxygen species and synaptic plasticity in the aging hippocampus. Ageing Res Rev 2004; 3: 431- 43.

Sherry JM, Crowe SF. Ouabain does not impair reconsolidation following a reminder of passive avoidance learning in the day-old chick. Neurosci Lett 2007; 423: 123-27.

Ticku MK, Burch T. Purine inhibition of [3H]-gamma-aminobutyric acid receptor binding to rat brain membranes. Biochem Pharmacol 1980; 29: 1217-20.

Visser JE, Bär PR, Jinnah HA. Lesch-Nyhan disease and the basal ganglia. Brain Res Bull 2000; 32: 449 -75.

White NM, McDonald RJ. Multiple parallel memory systems in the brain of the rat Neurobiol Learn Mem 2002; 77: 125 – 84.

Wyse ATS, Bavaresco CS, Reis EA, Zugno AI, Tagliari B, Calcagnotto T, et al. Training in inhibitory avoidance causes a reduction of Na^+, K^+ - ATPase activity in rat hippocampus. Physiol Behav 2004; 80: 475 – 79.

Figure 1. Effect of intrastriatal hypoxanthine infusion 30 min before training on step-down inhibitory avoidance task. (a) Data are median (interquartile range) of 9-11 animals in each group. *Different from the control group (Mann-Whitney; $p<0.05$).

Figure 2. Effect of intrastriatal hypoxanthine infusion immediately after training on step-down inhibitory avoidance task. (a) Data are median (interquartile range) of 11-12 animals in each group. *Different from the control group (Mann-Whitney; $p<0.05$).

Figure 3. Effect of intrastriatal hypoxanthine infusion 30 min before test session on step-down inhibitory avoidance task. (a) Data are median (interquartile range) of 8-9 animals in each group. *Different from the control group (Mann-Whitney; $p<0.05$).

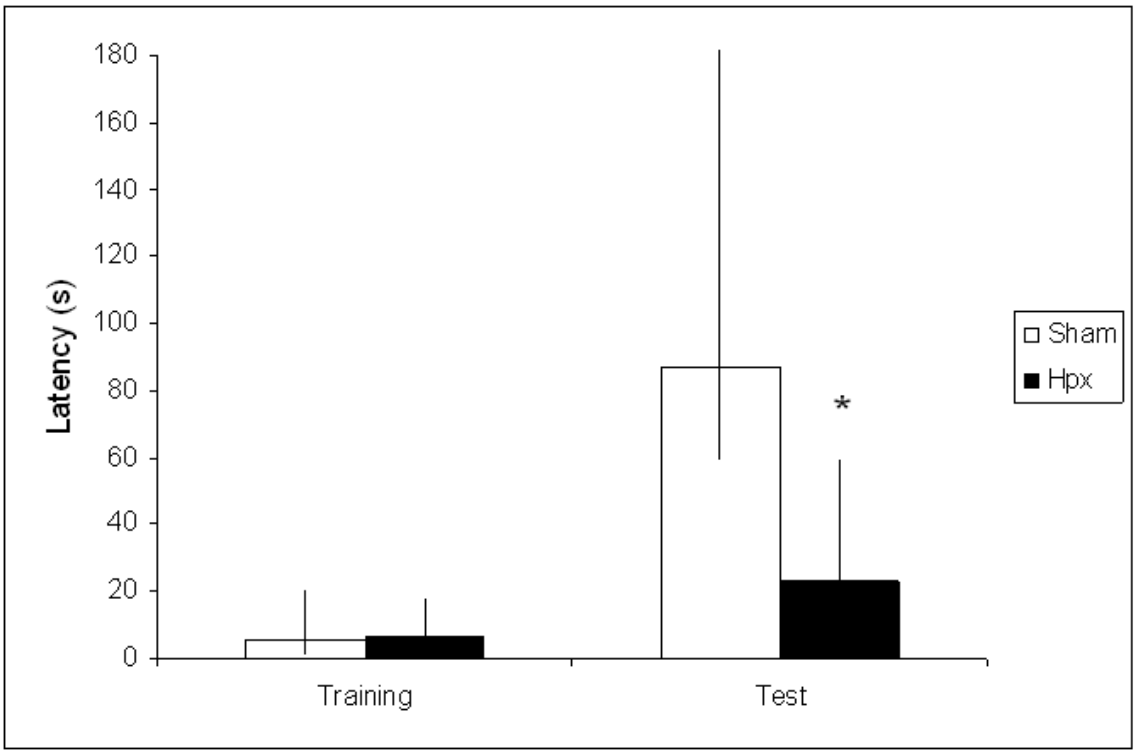

180
160
140
120
100
80
60
40
20
0
Latency (s)
Training
Test
*
Sham
Hpx

Figure 1

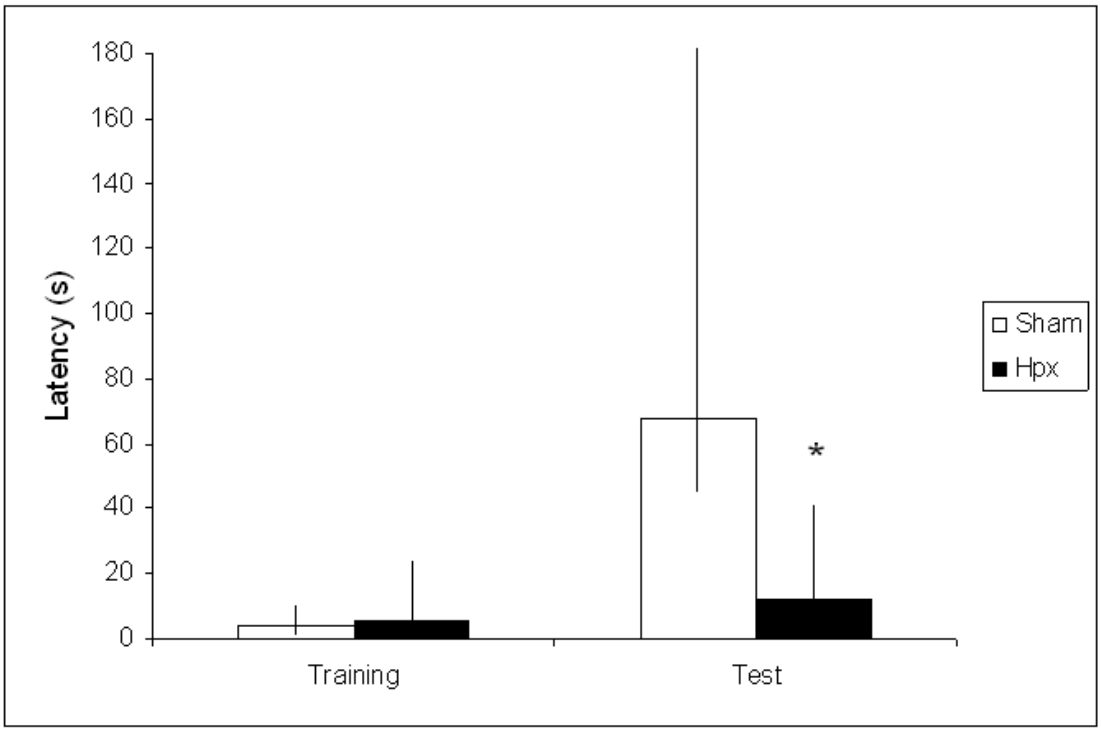

180
160
140
120
100
80
60
40
20
0
Latency (s)
Training
Test
*
Sham
Hpx

Figure 2

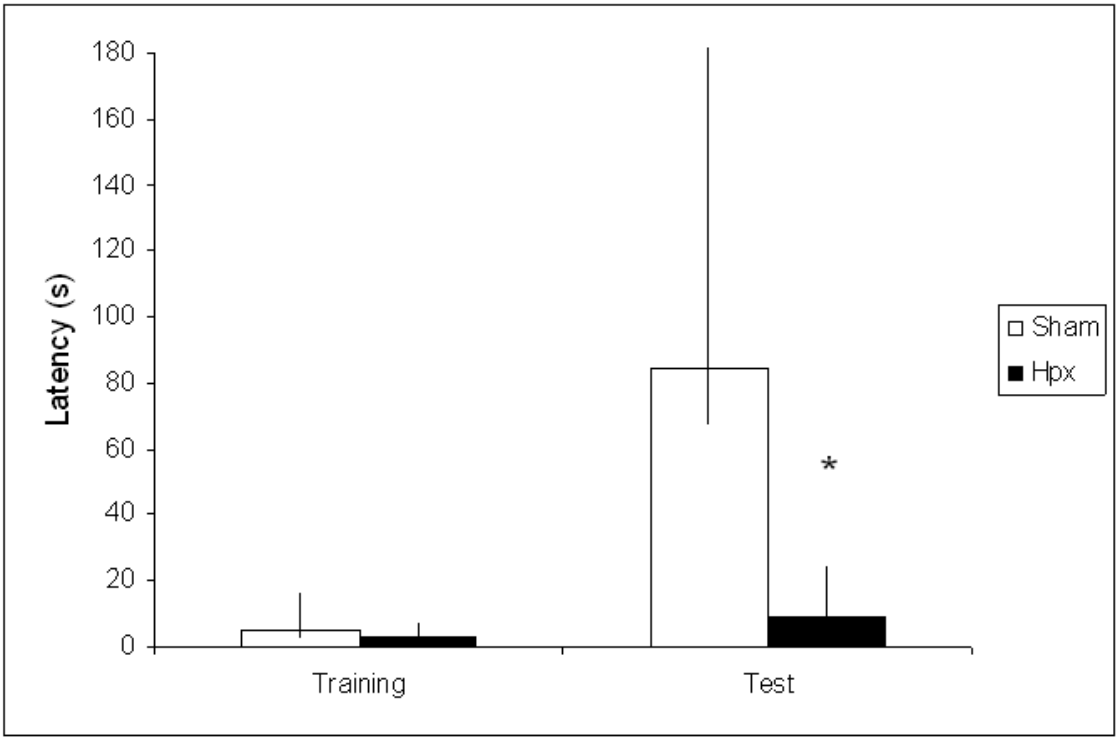

180
160
140
120
100
80
60
40
20
0
Latency (s)
Training
Test
*
Sham
Hpx

Figure 3

DISCUSSÃO

4. DISCUSSÃO

A síndrome de Lesch Nyhan é um erro inato do metabolismo das purinas, de característica recessiva, ligado ao sexo. Caracteriza-se, bioquimicamente, pela deficiência na atividade da enzima HGPRT, resultando em acúmulo tecidual, principalmente, de hipoxantina. Os pacientes portadores da síndrome são assintomáticos ao nascimento, desenvolvendo as primeiras manifestações clínicas da doença entre 3 e 6 meses de vida. O quadro clínico manifestado é bastante característico, incluindo, hiperuricemia, gota, alterações motoras e cognitivas, retardo mental, espasticidade e automutilação (NYHAN, 1978, VISSER at al., 2000, JINNAH & FRIEDMANN, 2001). Dados na literatura demonstraram redução seletiva do conteúdo dopaminérgico dos neurônios localizados no estriado, similares àqueles encontrados em pacientes portadores da doença de Parkinson (VISSER et al., 2002).

As terapias propostas para a síndrome de Lesch Nyhan apresentam um enfoque estritamente sintomático. Uso de fármacos a fim de prevenir as alterações renais e cerebrais tem sido descrito, todavia não apresenta eficácia absoluta (VISSER et al., 2000, DeANTONIO et al., 2002). As alterações relacionadas com o comportamento e, particularmente, as manifestações auto-destrutivas são de difícil tratamento, tanto que as alternativas terapêuticas consistem de terapia seguida de exercícios de relaxamento, autocontrole frente às situações estressantes e placas de mordida (OLSON & HOULIHAN, 2000). Neste contexto, faz-se necessário a execução de mais estudos a fim de elucidar a fisiopatologia da doença.

Considerando que a hipoxantina, principal metabólito acumulado na síndrome de Lesch Nyhan, vem sendo apontada como neurotoxina, neste trabalho desenvolvemos um

modelo experimetal que se baseia na infusão de hipoxantina diretamente no estriado, utilizando como recurso a cirurgia estereotáxica cujas coordenadas foram obtidas do atlas de coordenadas estereotáxicas desenvolvido por Paxinos e Watson (1986). Para tanto, 2 µL de uma solução de hipoxantina (20 pmol/ 2µl) ou veículo (salina) foram administrados no estriado direito do animal por um intervalo de 1 mim. A dose de hipoxantina utilizada em nosso estudo foi escolhida de acordo com Puig e colaboradores (1989), tentando-se obter concentrações semelhantes às encontradas em pacientes com Lesch Nyhan. O estriado foi utilizado como local para a administração da droga devido às evidentes lesões estriatais encontradas nos pacientes portadores da doença de Lesch Nyhan (VISSER at al., 2000, JINNAH & FRIEDMANN, 2001).

Utilizando este modelo, o primeiro objetivo do presente trabalho consistiu em avaliar o efeito da administração intra-estriatal de hipoxantina sobre a atividade da Na^+, K^+-ATPase de membrana plasmática sináptica de estriado, hipocampo e córtex cerebral de ratos adultos sacrificados 30 min após a infusão da droga.

Nossos resultados mostraram uma inibição significativa da atividade da Na^+, K^+-ATPase em estriado de ratos sacrificados 30 min (51%) após a infusão de hipoxantina. Em relação ao hipocampo e ao córtex cerebral, a atividade da enzima apresentou redução de 40% e 44% após a administração da droga, respectivamente. Esses resultados estão de acordo com trabalhos prévios, os quais demonstraram que hipoxantina *in vitro* é capaz de reduzir a atividade da Na^+, K^+- ATPase em membrana plasmática sináptica de estriado de ratos de 6 dias (BAVARESCO et al., 2004).

É importante salientar que o estriado, o hipocampo e o córtex cerebral configuram uma rede neuronal complexa e interligada (MAHON et al., 2004), fato este que poderia explicar a inibição da Na^+, K^+-ATPase nas três estruturas cerebrais após a administração intra-estriatal de hipoxantina. Nessa perspectiva, as alterações estriatais

encontradas neste trabalho podem estar alterando rotas bioquímicas no hipocampo e córtex cerebral através de circuitos neuronais específicos. Todavia, efeitos decorrentes da difusão de hipoxantina para o hipocampo e para o córtex cerebral não podem ser descartados.

As diferentes respostas evidenciadas nas três estruturas cerebrais podem estar relacionadas às variadas distribuições das formas isozímicas da Na^+, K^+-ATPase no tecido cerebral (SWEADNER, 1991). Neste contexto, Hieber e colaboradores (1991) demonstraram que o RNA mensageiro (mRNA) da subunidade alfa 1 é expresso, predominantemente, no estriado e hipocampo, enquanto que o córtex cerebral é rico em alfa 3. As características teciduais referentes à distribuição das subunidades alfa podem conferir níveis variados de susceptibilidade ao dano na Na^+, K^+-ATPase.

Dados na literatura demonstram que a atividade da enzima Na^+, K^+ - ATPase é inibida por radicais livres (LEES et al., 1993). Estes são, por definição, moléculas ou fragmentos de moléculas com um ou mais elétrons desemparelhados (HALLIWELL, 2006), os quais podem exercer seus efeitos nocivos através peroxidação de lipídios de membrana, inativação de enzimas através da oxidação dos grupamentos sulfidríla enzimáticos, despolimerização de polissacarídeos e ruptura de ácidos nucléicos. A interação entre a produção de radicais livres e as defesas antioxidantes fazem parte do metabolismo celular fisiológico e, alterações nesse sistema redox apresentam um grande papel no desenvolvimento das disfunções do SNC (CHOI, 1993, HALIWEEL, 2006).

Dando continuidade no nosso estudo, investigamos os possíveis mecanismos inibitórios provocados pela hipoxantina sobre a atividade da Na^+, K^+ - ATPase em estriado, hipocampo e córtex cerebral, avaliando parâmetros de estresse oxidativo, tais como a quimioluminescência (medida de LPO), TRAP (medida da capacidade antioxidante tecidual) e o conteúdo total de grupos tiólicos reduzidos. Nossos resultados

mostraram um aumento significante da quimioluminescência, bem como uma redução no TRAP 30 min após a injeção de hipoxantina, nas três estruturas cerebrais avaliadas. Não observamos nenhuma alteração em relação ao conteúdo total de grupos tiólicos reduzidos. Deste modo, nossas evidências indicam o envolvimento da peroxidação lipídica e/ou a redução das defesas antioxidantes teciduais na diminuição da atividade da Na^+, K^+ - ATPase encontrada neste estudo.

Considerando os resultados citados no capítulo I, posteriormente investigamos o efeito da administração intra-estriatal de hipoxantina sobre as atividades da Na^+, K^+-ATPase, AChE e CAT, em diferentes períodos após a infusão da droga no estriado, hipocampo e córtex cerebral de ratos.

Nossos resultados mostraram uma inibição da atividade Na^+, K^+- ATPase em estriado de ratos sacrificados 3 h (45%), 24 h (52%) e 7 dias (71%) após a realização da administração de hipoxantina. Em relação ao hipocampo, a atividade da enzima apresentou-se significativamente reduzida 3 h (44%), 24 h (42%) e 7 dias (59%) após a infusão da droga. No córtex cerebral não foi observada nenhuma alteração na atividade enzimática 3 h, 24 h ou 7 dias após a administração de hipoxantina.

Autores indicam que a atividade da Na^+, K^+-ATPase pode ser regulada de forma diferencial ao longo do tempo. As alterações de longa duração incluem modificações na taxa de síntese/degradação enzimáticas, enquanto que as de curta duração são referenciadas como modificações rápidas e dinâmicas na enzima, mediada, muitas vezes, por reações de fosforilação, ligação direta de compostos em sítios enzimáticos, bem como alterações na fluidez de membrana (BERTORELLO & KATS, 1995). Desta forma, a inibição da atividade da Na^+, K^+-ATPase ao longo do período experimental realizado no nosso trabalho (30 min, 3 h e 24 h e 7 dias) parece envolver diferentes processos celulares.

Em relação à atividade da catalase, observamos significativa redução (25%) da atividade enzimática, bem como o aumento (18%) na atividade em estriado de ratos 30 min e 7 dias após a infusão de hipoxantina, respectivamente. No hipocampo, a atividade da catalase apresentou-se reduzida 24 h (33%) e 7 dias (32%) pós-infusão de hipoxantina. Além disso, a atividade enzimática foi inibida nos 30 min (20%) e nas 3 h (20%) posteriores a injeção de hipoxantina em córtex cerebral de ratos.

Embora os exatos mecanismos envolvidos nos efeitos mediados pela hipoxantina sobre a catalase mereçam estudos adicionais, sugerimos que a redução da atividade enzimática e subseqüente elevação ocorram através de mecanismos compensatórios decorrentes do aumento na síntese de proteína na tentativa de sobrepor o desafio metabólico. Neste contexto, evidências indicam que as enzimas antioxidantes podem a responder aos danos mediados pelo estresse oxidativo através do aumento na transcrição gênica (TRAVACIO & LLESUY, 1996). Assim, o aumento na atividade da catalase observada 7 dias após a administração de hipoxantina em estriado pode estar relacionada a adaptação enzimática decorrente da indução do estresse oxidativo.

A atividade da acetilcolinesterase, outra enzima responsável pela homeostase cerebral, também foi avaliada. Para tanto, os animais foram sacrificados 30 min, 3 h e 24 h, bem como 7 dias após a administração intra-estriatal de hipoxantina. Os resultados indicaram uma redução da atividade as acetilcolinesterase em estriado (3 h e 24 h), aliada ao aumento da atividade enzimática em córtex cerebral (24 h) após a infusão dessa oxipurina, não havendo, contudo, alteração na atividade da mesma em hipocampo. Nossos resultados estão em concordância com outras evidências da literatura que apontam para alterações no sistema colinérgico em pacientes com Lesch Nyhan (SAITO e TAKASHIMA, 2000).

Considerando os resultados previamente discutidos neste trabalho, os quais mostraram que a administração intra-estriatal de hipoxantina inibe a atividade da Na^+, K^+ - ATPase, bem como parece induzir o estresse oxidativo em cérebro de ratos e, que estudos prévios indicam a capacidade de agentes antioxidantes em prevenir a inibição dessa enzima (BAVARESCO et al., 2005), também investigamos a influência do pré-tratamento com as vitaminas E e C sobre os efeitos mediados pela infusão intra-estriatal de hipoxantina sobre as atividades da Na^+, K^+ - ATPase, SOD, CAT e GPx em estriado de ratos. Avaliamos, além disso, a influência do pré-tratamento com as vitaminas E e C sobre as alterações provocas pela hipoxantina nos parâmetros de estresse oxidativo, denominados TBARS e TRAP na mesma estrutura cerebral.

Nesta perspectiva, alguns autores observaram que a inibição da Na^+, K^+ - ATPase pode ser prevenida por antioxidantes *in vitro* (VIETTA et al., 1996; STRECK et al., 2001, WYSE et al., 2002, FRANZON et al, 2003). Um mecanismo eficaz de proteção antioxidante consiste na transferência de elétrons aos compostos reativos para saturar suas afinidades eletrônicas. A fim de manter o equilíbrio redox celular, é necessária a presença de um meio intracelular capaz de prover equivalentes de redução (CRUZ et al., 2003).

Evidências mostram que a glutationa possui um papel importante na neutralização das espécies reativas de oxigênio (WARNER et al., 2004). Apesar de a glutationa reagir espontaneamente com radicais livres, sua maior eficácia antioxidante está associada às enzimas glutationa peroxidase, glutationa redutase e glutationa transferase (CRUZ et al., 2003). Aliada a ação da glutationa, o α – tocoferol (vitamina E) atua bloqueando a propagação da cascata de peroxidação ao longo da membrana celular (CHOI, 1993). Alguns autores sugerem um papel preventivo e terapêutico do α – tocoferol em doenças neurodegenerativas, tais como as doenças de Parkinson e de Alzheimer (CHOI, 1993,

LOPEZ & BECKER, 2002, BEAL, 2002). Aliado a isto, evidências apontam para a participação do ácido ascórbico (vitamina C) nos mecanismos envolvidos na redução do α-tocoferol (KELLY, 1998; FREI, 1999).

O cérebro é, particularmente, suscetível ao estresse oxidativo, uma vez que contém grandes quantidades de lipídios insaturados e apresenta um alto consumo de oxigênio proveniente de sua alta taxa metabólica (HALLIWELL, 1996; FLOYD, 1999; HALLIWELL, 2006). O estriado, por sua vez, apresenta grandes quantidades de ferro e melanina, os quais podem catalisar a produção de radical hidroxila frente a processos oxidativos (VISSER et al., 2002). Além disso, o cérebro apresenta baixos níveis de defesas antioxidantes enzimáticas e não-enzimáticas (REZNICK & PACKER, 1993, CHOI, 1993, HALLIWELL, 1996; FLOYD, 1999). Neste sentido, a adoção de medidas preventivas em relação aos danos oxidativos ao cérebro é de extrema importância.

Para a realização desta metodologia experimental, os animais foram pré-tratados através de injeções diárias com as vitaminas E e C por um período de 7 dias (WYSE et al., 2000). No quinto dia de pré-tratamento, os animais foram submetidos à cirurgia estereotáxica (PAXINOS & WATSON, 1986). A administração de hipoxantina ou salina ocorreu 12 h após a última injeção de vitaminas. Para a determinação de todos os parâmetros experimentais, os animais foram sacrificados 30 min após a administração de hipoxantina.

Inicialmente, investigamos a influência das vitaminas E e C sobre a atividade da Na^+, K^+ - ATPase. Após análise estatística dos dados, foi possível evidenciar um efeito preventivo das vitaminas sobre a atividade da Na^+, K^+ - ATPase. Estes resultados estão de acordo com dados prévios que mostraram o efeito protetor da glutationa e do trolox sobre a inibição da Na^+, K^+ - ATPase provocada pela hipoxantina *in vitro* (BAVARESCO et al., 2005). Além disso, uma vez que não observamos alterações nos

conteúdo de grupos tiólicos totais reduzidos em presença de hipoxantina, é possível inferir que a inibição da Na^+, K^+ - ATPase não ocorra através da oxidação dos grupos tiólicos presentes na enzima. Contudo, em virtude da ação da vitamina E sobre os lipídios de membrana, é possível que as vitaminas E e C atuem inibindo a peroxidação de lipídios e/ou a geração de radicais livres induzidos pela hipoxantina.

Aliado a este fato, os resultados também mostraram o efeito protetor das vitaminas sob as alterações no TBARS, sugerindo que a ação protetora das vitaminas E e C ocorra através da inibição na geração de radicais livres e/ou na lipoperoxidação. Por outro lado, as vitaminas E e C não preveniram a redução do TRAP. Tendo em vista que o principal antioxidante medido na técnica do TRAP é o GSH (EVELSON et al., , 2001), este resultado corrobora com a indicação que as vitaminas E e C atuem inibindo a peroxidação de lipídios e/ou a geração de radicais livres induzidos pela hipoxantina.

Em relação à inibição atividade das enzimas SOD e CAT, mediada pela administração de hipoxantina, é possível observar um efeito protetor das vitaminas sobre as atividades destas duas enzimas. Por outro lado, a infusão intra-estriatal de hipoxantina aumentou a atividade da enzima GPx em estriado de ratos. Embora não saibamos exatamente qual o mecanismo envolvido na ativação da GPx, sugere-se um possível efeito compensatório decorrente da inibição da atividade da CAT. Entretanto, diversos autores indicam a ação redutora da GPx sobre os lipoperóxidos (MICHIELS et al., 1994). Partindo da observação que hipoxantina aumenta o TBARS, um índice de peroxidação lipídica, especula-se que o aumento na atividade da GPx seja decorrente de uma estratégia celular para reduzir o dano oxidativo mediado pela hipoxantina no estriado.

Dando seqüência em nossos estudos, avaliamos o efeito da administração de hipoxantina sobre a hidrólise do ATP, ADP e AMP em sinaptossoma de estriado de

ratos. Neste contexto, algumas purinas são reconhecidas como importantes neuromoduladores, assim, além do papel energético do ATP, os produtos advindos da sua hidrólise (ADP, AMP e adenosina) também apresentam função celular sinalizadora (KOMOSZYNSKI & WOJTCZAK, 1996). Neste contexto, evidências na literatura indicam o efeito regulatório da adenosina sobre o sistema de monoaminas (ZHU et al., 2007), bem como nas alterações motoras e comportamentais, tais como a agressividade, através da ação em seus receptores (RIBEIRO et al., 2003).

As concentrações extracelulares de adenosina dependem da degradação do ATP, ADP e AMP através da ação catalítica de enzimas da família das ectonucleotidases, tais como a ATP-difosfohidrolase (apirase; NTPDases) e a ecto-5´-nucleotidase (ZIMMERMANN, 2006). A apirase é responsável pela hidrólise extracelular do ATP e ADP, resultando em uma molécula de AMP. Em contrapartida, a enzima ecto-5 ´-nucleotidase é responsável pela hidrólise do AMP até adenosina (ZIMMERMANN, 2006).

Considerando que evidências apontam para o envolvimento da adenosina nos mecanismos de dano cerebral observados em pacientes com Lesch Nyhan (PRIOR et al., 2006), neste trabalho também avaliamos o efeito da administração intra-estriatal de hipoxantina sobre as atividades das enzimas ATP difosfoidrolase (apirase) e 5 ´-nucleotidase em estriado de ratos adultos. O estudo do efeito *in vitro* da hipoxantina sobre as atividades enzimáticas na mesma estrutura cerebral também foi realizado. Baseando-se nas evidências que indicam o envolvimento característico do estriado em pacientes com Lesch Nyhan (JINNAH & FRIEDMANN, 2001), utilizamos, nos estudos bioquímicos subseqüentes, o estriado como região cerebral alvo para nosso estudo.

Para a verificação dos efeitos da hipoxantina, os animais foram sacrificados 30 min, 24 h e 7 dias após a administração da droga. Nossos resultados mostraram, 24 h após

infusão intra-estriatal de hipoxantina, redução significativa na hidrólise do ATP (35%), ADP (27%) e AMP (29%) em estriado de ratos, contudo tal efeito sobre a hidrólise dos nucleotídeos não foi observado 30 minutos e 7 dias após a administração de hipoxantina. Por outro lado, nossos resultados mostraram que hipoxantina *in vitro* não é capaz de inibir as atividades das enzimas ATP difosfoidrolase (apirase) e 5´-nucleotidase, indicando que a inibição das atividades enzimáticas causada pela hipoxantina não é decorrente de um efeito direto dessa oxipurina sobre as enzimas. Neste contexto, estudos sobre possíveis mecanismos indiretos decorrentes da infusão intra-estriatal de hipoxantina são necessários.

Estudos na literatura indicam que inibição da hidrólise do ATP e ADP pode desencadear um efeito em cascata, culminando com a inibição estequiométrica das enzimas ATP difosfoidrolase (apirase) e 5´-nucleotidase, as quais atuam em conjunto na manutenção dos níveis extracelulares de ATP, ADP, AMP e adenosina (VUADEN et al., 2007). A defosforilação dos nucleotídeos da adenina, através da ação das ectonucleotidases, pode inativar a ação neurotransmissora de tais nucleotídeos através da conversão em produtos inativos, ou de forma indireta, através da ação de compostos sobre as atividades das enzimas associadas à cascata das ectonucleotidases (ZIMMERMANN, 2006).

Pesi e colaboradores (2000) demonstraram aumento na atividade da enzima 5 ´-nucleotidase citosólica em eritrócitos de paciente portadores desta doença. Além disso, a alteração deletéria provocada pela hipoxantina no transporte de adenosina em linfócitos foi recentemente proposta (TORRES et al., 2004). Neste sentido, a inibição na cadeia de hidrólise dos nucleotídeos mediada pela hipoxantina pode provocar redução das concentrações extracelulares de adenosina, molécula reconhecidamente neuroprotetora (ZIMMERMANN, 2006). Neychev e Mitev (2004) discutiram o papel da

adenosina nas alterações comportamentais, como a auto-mutilação, em pacientes com Lesch Nyhan. Além disso, recente trabalho de Torres e colaboradores (2004) sugeriu que o excesso de hipoxantina no estriado pode desencadear o processo de auto-mutilação através da sua ação modulatória nas concentrações de adenosina extracelular. Nesta perspectiva, a redução da hidrólise do AMP observado no presente trabalho, desencadeada pela administração de hipoxantina, poderia culminar com a redução paralela nos níveis de adenosina e assim aumentar a susceptibilidade neuronal ao dano, bem como alterar alguns parâmetros comportamentais.

Não obstante, devemos relembrar que 7 dias após a infusão de hipoxantina, as atividades das enzimas ATP difosfoidrolase (apirase) e 5´-nucleotidase retornaram aos valores basais, sugerindo um mecanismo de adaptação enzimática. Neste sentido, evidências sugerem que as ectonucleotidases podem responder às alterações em suas atividades através do controle em sua transcrição gênica (SALEM & TRAMS, 1983). A partir das observações que sugerem um mecanismo gênico modulatório sobre as atividades das referidas enzimas e tendo em vista a ação inibitória da hipoxantina, 24 h após a sua administração, sobre as atividades das ectonucleotidases em estriado, decidimos investigar a expressão relativa das NTPDases (NTPDase 1, NTPDase 2 e NTPDase 3) assim como da ecto-5´-nucleotidase através da técnica do RT-PCR, a qual baseia-se na obtenção do cDNA através do mRNA obtido no processamento da amostra pela ação da enzima transcriptase reversa (FERNANDES & SKIENA, 2007), utilizando como controle da técnica a expressão de ß-actina. É importante evidenciar que as NTPDases enfocadas neste estudo estão relacionadas com o sistema nervoso central (SNC) e diferem entre si na preferência pelo substrato (NTPDase 1, NTPDase e NTPDase 3) (NEDELJKOVIC et al., 2006).

Nossos resultados mostraram que a administração intra-estriatal de hipoxantina não alterou as expressões relativas das NTPDases estudadas, todavia acarretou um aumento (80%) na expressão relativa da ecto-5´-nucleotidase. Embora não saibamos os exatos mecanismos que resultaram no aumento da expressão enzimática, é possível que mecanismos transcripcionais compensatórios decorrentes da inibição da atividade enzimática regulem este processo, no intuito de estabelecer os níveis de adenosina. Em concordência com nossos resultados, Salem e Trams (1983) sugeriram que as células podem responder à inativação da ecto-5´-nucleotidase através da reposição de 80% da atividade enzimática dentro de 24 h em cultura de glioblastoma.

Levando em consideração a discussão prévia estabelecida sobre o papel dos radicais livres no SNC, na inibição das atividades da ATP difosfoidrolase (apirase) e 5 ´-nucleotidase provocada pelo estresse oxidativo (DELWING et al., 2005), e nas evidências apresentadas previamente descritas em relação à participação do estresse oxidativo nas alterações bioquímicas cerebrais mediadas pela hipoxantina, também estudamos o envolvimento da formação de radicais livres, LPO e/ou estresse oxidativo na inibição das atividades da ATP difosfoidrolase (apirase) e 5´-nucleotidase causada pela hipoxantina, utilizando a técnica do TBARS. Nossos resultados demonstraram um aumento do TBARS em estriado de ratos, 24 h após a administração de hipoxantina. Neste contexto, é provável que o efeito inibitório da hipoxantina sobre as atividades das ectonucleotidases envolva a participação da LPO e/ou indução de estresse oxidativo.

A influência do pré-tratamento com as vitaminas E e C sobre a hidrólise do ATP, ADP e AMP, bem como sobre o TBARS em estriado de ratos também foi avaliada. Os resultados mostraram que o pré-tratamento com as vitaminas E e C previniu a inibição das atividades das enzimas ATP difosfoidrolase (apirase) e ecto-5´-nucleotidase em sinaptossoma de estriado de ratos bem como o aumento no TBARS. Neste contexto, e

corroborando com os dados obtidos no capítulo III, é possível inferir que as vitaminas E e C estejam atuando sobre a peroxidação de lipídios e/ou sobre a geração de radicais livres induzidos pela hipoxantina.

Considerando as alterações neuroquímicas induzidas pela administração de hipoxantina neste modelo experimental em ratos e que evidências na literatura correlacionam o aumento nos níveis de hipoxantina com déficits cognitivos (ÄGREN et al., 1983), a próxima etapa deste trabalho foi investigar o papel da administração de hipoxantina sobre a memória/ aprendizagem de ratos na tarefa do labirinto aquático de Morris e esquiva inibitória. Não obstante, a fim de evidenciar a influência da administração de hipoxantina sobre a atividade motora dos animais, realizamos a tarefa de campo aberto.

Nossos resultados mostraram que a administração de hipoxantina provocou prejuízo na aprendizagem/memória, na memória de referência, na tarefa do labirinto aquático de Morris, bem como na latência para cruzar, pela primeira vez, sobre a plataforma no dia do teste. Em relação à memória de trabalho, semelhante prejuízo nos processo de memória/aprendizagem espacial também foram evidenciados. Todavia, nossos resultados não podem ser atribuídos a um possível prejuízo motor nos animais tratados com hipoxantina, uma vez que a velocidade dos animais durante o nado não foi diferente entre os grupos. Em concordência com esse dado, após a análise dos parâmetros experimentais avaliados na tarefa do campo aberto, sugere-se que a hipoxantina não altera o componente motor no animal, uma vez que não houve diferenças estatísticas entre o grupo controle e os animais tratados com hipoxantina.

Evidências da literatura apontam que lesões estriatais são capazes de alterar a memória espacial em ratos (MARTEL et al., 2007; De LEONIBUS et al., 2007). É importante salientar que evidências mostram que as regiões cerebrais agem

conjuntamente a fim de garantir os processos de formação da memória (IZQUIERDO et al., 2006). Neste contexto, autores indicam processos cooperativos entre o hipocampo e o estriado (principalmente o região medial do caudado-putâmen) nos sistemas bioquímicos correlacionados com a memória (IZQUIERDO et al., 2006; MARTEL et al., 2007). Todavia, é possível que ocorra mudança entre os sistemas paralelos relacionados aos mecanismos da memória dependente da tarefa comportamental a ser utilizada (ROSSATO et al., 2006). Neste caso, a utilização da tarefa de esquiva inibitória parece ter grande relevância para elucidarmos os mecanismos envolvidos no déficit de memória apresentado nos animais submetidos à infusão de hipoxantina.

Considerando o prejuízo na memória apresentado pelos animais submetidos em nosso modelo experimental, o presente trabalho buscou avaliar o conteúdo estriatal de monoaminas, tais como a dopamina (DA), ácido 3,4-dihidroxi-indolacético (DOPAC), ácido homovalínico (HVA), serotonina (5-HT) e de ácido 5-hidroxi-indolacético (5-HIAA) em ratos submetidos à administração intra-estriatal de hipoxantina. Para tal determinação, os animais foram sacrificados 7 dias após a infusão das drogas. Após a análise estatística dos dados, não foi encontrada nenhuma alteração nos níveis de DA, DOPAC e HVA, no entanto foi observada uma redução significativa do conteúdo estriatal de 5-HT e 5-HIAA, em animais submetidos à administração de hipoxantina. Neste contexto, estudos prévios evidenciaram a relação entre a redução dos níveis de 5-HIAA e prejuízos de aprendizagem/memória (BOLLA et al., 1998). Além disso, Lidberg e colaboradores (1984) encontraram redução nos níveis de 5-HT e 5-HIAA no fluido cerebroespinhal de pacientes com comportamento agressivo. Neste contexto, especula-se que o decréscimo nos níveis 5-HT e 5-HIAA provocados pela infusão de hipoxantina possam estar relacionados, pelo menos em parte, com a alteração comportamental observada na doença de Lesch Nyhan.

Com o intuito de explorar um pouco mais os aspectos comportamentais dos animais neste estudo, conforme descrito no capítulo VI, a tarefa de esquiva inibitória também foi utilizada em nosso estudo. Os resultados obtidos através da tarefa de esquiva inibitória também indicaram um prejuízo na aprendizagem/ memória, mediada pela hipoxantina. Nessa tarefa, a hipoxantina foi administrada 30 min antes do treino, imediatamente após o treino e 30 min antes do teste, a fim de estudarmos os efeitos da hipoxantina na aquisição, consolidação e evocação da memória, respectivamente. Nossos resultados demonstraram significativa redução na latência na esquiva inibitória nos animais tratados com hipoxantina 30 min antes do treino, imediatamente após o treino e 30 min antes do teste, indicando déficit de memória/ aprendizado mediado pela administração de hipoxantina.

Neste momento é importante evidenciar que a modulação da atividade da enzima Na^+, K^+ - ATPase parece influenciar os mecanismos de memória/aprendizado. Wyse e colaboradores (2004) mostraram redução significativa na atividade da enzima Na^+, K^+ - ATPase em animais submetidos a tarefa de esquiva inibitória. Além disso, autores demonstram que a administração de ouabaína, inibidor específico da atividade da Na^+, K^+ - ATPase é capaz de alterar a fase de consolidação da memória (GIBBS et al., 2003; SHERRY e CROWE, 2007). Neste contexto, é possível correlacionar à inibição da atividade da Na^+, K^+ - ATPase causada pela administração de hipoxantina com o prejuízo de memória observados nestes animais. Todavia a indução de estresse oxidativo (SERRANO e KLANN, 2004, MATTÉ et al. 2007), também parece estar envolvida em déficits de memória e pode contribuir para o déficit de memória observado nas tarefas do labirinto aquático de Morris e na esquiva inibitória.

Em suma, os achados do presente trabalho sugerem que a inibição das atividades das enzimas Na^+, K^+ - ATPase, AChE, ATP-difosfohidrolase e 5´-nucleotidase, a indução de

estresse oxidativo bem como as alterações comportamentais provocadas pela administração intra-estriatal de hipoxantina podem estar relacionados, pelo menos em parte, com a disfunção neurológica encontrada em pacientes com Lesch Nyhan.

CONCLUSÕES

5. CONCLUSÕES

1. A administração intra-estriatal de hipoxantina inibiu significativamente as atividades das enzimas Na^+, K^+ - ATPase , AChE, ATP-difosfohidrolase e ecto-5 ´-nucleotidase em cérebro de ratos. A expressão da enzima ecto-5´-nucleotidase estava aumentada 24 h após a administração de hipoxantina.

2. A infusão de hipoxantina aumentou a LPO (quimiluminescência, TBARS) e reduziu a capacidade antioxidante do tecido (TRAP), mas não alterou o conteúdo total de grupos tiólicos reduzidos em cérebro de ratos. Houve uma administração inibição nas atividades das enzimas SOD e CAT e um aumento na atividade da enzima GPX em cérebro de ratos.

3. O pré-tratamento com as vitaminas E e C foi capaz de reverter a inibição da Na^+, K^+ - ATPase, ATP-difosfohidrolase, 5´-nucleotidase, SOD, CAT e o aumento da GPx e do TBARS, mas não foi capaz de reverter a redução do TRAP provocada pela administração de hipoxantina em estriado de ratos.

4. A administração intra-estriatal de hipoxantina prejudicou significativamente a memória/ aprendizagem de ratos nas tarefas do labirinto aquático de Morris e na tarefa de esquiva inibitória, porém não provocou déficit motor em ratos submetidos à tarefa do campo aberto.

5. A administração intra-estriatal de hipoxantina reduziu significativamente o conteúdo de serotonina, bem como do ácido 5-hidroxi-indolacético em estriado de ratos, porém não alterou o conteúdo de dopamina, ácido 3,4-dihidroxi-indolacético e do ácido homovalínico (HVA) em estriado de ratos.

A utilização de modelos animais para o estudo de doenças cerebrais tem se mostrado uma excelente método para melhorar a compreeensão da patofisiologias das doenças humanas. Neste contexto, o modelo desenvolvido no nosso trabalho possibilitou o estudo dos efeitos cerebrais da hipoxantina, principal metabólito acumulado na síndrome de Lesch Nyhan. Considerando nossos achados em conjunto, é possível que a inibição na atividade das enzimas Na^+, K^+ - ATPase, AChE, ATP-difosfohidrolase e 5 ´-nucleotidase e/ou indução do estresse oxidativo, aliado ao prejuízo de memória/ aprendizagem observado em animais tratados com hipoxantina possam estar envolvidos nos mecanismos pelos quais a hipoxantina é neurotóxica. Acreditamos que nossos resultados possam contribuir, pelo menos em parte, para a compreensão da disfunção neurológica encontrada em pacientes portadores da síndrome de Lesch-Nyhan.

PERSPECTIVAS

6. PERSPECTIVAS

6.1. Realizar estudos ontogenéticos sobre o efeito da administração intra-estriatal em parâmetros bioquímicos, tais como a atividade da Na^+, K^+ - ATPase e o estresse oxidativo em estriado de ratos.

6.2. Verificar o efeito da administração intra-estriatal de hipoxantina sobre o metabolismo energético em estriado de ratos.

6.3. Verificar o efeito da co-administração intra-estriatal de 6-hidroxidopamina e hipoxantina sobre a atividade da Na^+, K^+ - ATPase, sobre parâmetros de estresse oxidativo e metabolismo energético em estriado de ratos.

6.4. Verificar o efeito *in vitro* do Z-nucleotídeo, outra substância acumuladas na doença de Lesch-Nyhan, sobre a atividade da Na^+, K^+ - ATPase, estresse oxidativo e metabolismo energético em estriado de de ratos.

REFERÊNCIAS BIBLIOGRÁFICAS

7. REFERÊNCIAS BIBLIOGRÁFICAS

ÄGREN, H., NIKLASSON, F., HÄLLGREN, R. Brain purinergic activity linked with depressive symptomatology: hypoxanthine and xanthine in CSF of patients with major depressive disorders. Psychiatry Res. 9 :179-189, 1983.

BALIS, M.E. Uric acid metabolism in man. Adv. Clin. Chem. 18: 213-246, 1976.

BATTASTINI, A.M., OLIVEIRA, E.M., MOREIRA, C.M., BONAN, C.D., SARKIS, J.J., DIAS, R.D. Solubilization and characterization of an ATP diphosphohydrolase (EC 3.6.1.5) from rat brain synaptic plasma membranes. Biochem. Mol. Biol. Int. 37(2):209-219, 1995.

BAVARESCO, C.S., ZUGNO, A.I., TAGLIARI, B., WANNMACHER, C.M., WAJNER, M., WYSE, A.T. Inhibition of Na^+, K^+-ATPase activity in rat striatum by the metabolites accumulated in Lesch-Nyhan disease. Int. J. Dev. Neurosci. 22(1):11-17, 2004.

BAVARESCO, C.S., CHIARANI, F., MATTÉ, C., WAJNER, M., NETTO, C.A., DE SOUZA WYSE, A.T. Effect of hypoxanthine on Na+,K+-ATPase activity and some parameters of oxidative stress in rat striatum. Brain Res. 1041: 198-204, 2005.

BEAL, M.F. Oxidatively modified proteins in aging and disease. Free. Radic. Biol. Med. 32: 797 – 803, 2002.

BECKMAN, J.S., LIU, T.H., HOGAN, E.L., FREEMAN, B.A., HSU, C.Y. Oxygen free radicals and xanthine oxidase in cerebral ischemic injury in the rat. Soc. Neurosci. Abstr. 13: 1498, 1987.

BERTELLI, M., CECCHIN, S., LAPUCCI, C., JACOMELLI, G., JINNAH, H.A., PANDOLFO, M., MICHELI, V. Study of the adenosinergic system in the brain of

HPRT knockout mouse (Lesch-Nyhan disease). Clin Chim Acta. 373(1-2): 104-107, 2006.

BERTORELLO, A.M. & KATZ, A.I. Regulation of Na^+,K^+- pump activity: pathways between receptors and effectors. NIPS. 10:253-259, 1995.

BICKEL, H. Early diagnosis and treatment of inborn errors of metabolism. Enzyme. 38: 14-26, 1987.

BLANCO, G. & MERCER, R. Isozymes of the Na^+,K^+-ATPase: heterogeity in structure, diversity in function. Am. J. Physiol. 275: F633-F650, 1998.

BOLLA, K.I., MCCANN, U.D., AND RICAURTE, G.A. Memory impairament in abstinent MDMA ("Ecstasy") users. Neurology 51: 1529-1530, 1998.

BOLDYREV, A.A. Na^+,K^+-ATPase. In: Science and Technology, vol. 17. pp. 5-120, 1985.

BÖHMER, A.E., STRECK, E.L., STEFANELLO, F., WYSE, A.T., SARKIS, J.J. NTPDase and 5'-nucleotidase activities in synaptosomes of hippocampus and serum of rats subjected to homocysteine administration. Neurochem Res. 29(7): 1381-1386, 2004.

BONAN, C.D., DIAS, M.M., BATTASTINI, A.M., DIAS, R.D., SARKIS, J.J. Inhibitory avoidance learning inhibits ectonucleotidases activities in hippocampal synaptosomes of adult rats. Neurochem Res. 23(7): 977-982, 1998.

BONAN, C.D., ROESLER, R., QUEVEDO, J., BATTASTINI, A.M., IZQUIERDO, I., SARKIS, J.J. Effects of suramin on hippocampal apyrase activity and inhibitory avoidance learning of rats. Pharmacol Biochem Behav. 63(1): 153-158, 1999.

BRUEL-JUNGERMAN, E., DAVIS, S., LAROCHE, S. Brain plasticity mechanisms and memory: a party of four. Neuroscientist. 13(5): 492-505, 2007. Review.

BRUNO, A.N., DINIZ, G.P., RICACHENEVSKY, F.K., POCHMANN, D., BONAN, C.D., BARRETO-CHAVES, M.L., SARKIS, J.J. Hypo-and hyperthyroidism affect the ATP,

ADP and AMP hydrolysis in rat hippocampal and cortical slices. Neurosci. Res. 52(1): 61-68, 2005.

BULL, M. & LaVECCHIO, F. Behavior therapy for a child with Lesch-Nyhan syndrome. Develop. Med. Child. Neurol. 20: 368-375, 1978.

CAMACHO, A. & MASSIEU, L. Role of glutamate transporters in the clearance and release of glutamate during ischemia and its relation to neuronal death. Arch. Med. Res. 37(1): 11-18, 2006.

CAMMAROTA, M., BEVILAQUA, L.R., ROSSATO, J.I., RAMIREZ, M., MEDINA, J.H., IZQUIERDO, I. Relationship between short- and long-term memory and short- and long-term extinction. Neurobiol Learn Mem. 84(1): 25-32, 2005.

CAPELLA, L.S., GEFE, M., SILVA, E.F., MORALES, M.M, AFFONSO-MITDIERI, O., LOPES, A.G., RUMJANEK, V.M., CAPELLA, M.A. Reduced glutathione protect cells from ouabain toxicity. Biochim. Biophys. Acta. 1526: 293-300, 2001.

CAUWELS, R.G. & MARTENS, L.C. Self-mutilation behaviour in Lesch-Nyhan syndrome. J Oral Pathol Med. 34(9): 573-575, 2005.

CHAN, K.M., DELFERT, D., JUNGER, K.D. A direct colorimetric assay for Ca^{+} -stimulated ATPase activity. Anal. Biochem**.** 157: 375-380, 1986.

CHEN, C.M., LIU, S.H., LIN-SHIAU, S.Y. Honokiol, a neuroprotectant against mouse cerebral ischaemia, mediated by preserving Na+, K+-ATPase activity and mitochondrial functions. Basic Clin. Pharmacol. Toxicol. 101(2):108-116, 2007.

CHOI, B.H. Oxygen, antioxidants and brain dysfunction. Yonsei. Med.J. 33: 1-10, 1993.

COGNATO, G.DE P., BRUNO, A.N., DA SILVA, R.S., BOGO, M.R., SARKIS, J.J., BONAN, C.D. Antiepileptic drugs prevent changes induced by pilocarpine model of epilepsy in brain ecto-nucleotidases. Neurochem. Res. 32(6):1046-1055, 2007.

CORNELIUS, F. & MAHMMOUD, Y.A. Functional modulation of the sodium pump: the regulatory proteins “fixit”. News. Physiol. Sci. 18: 119-124, 2003.

CORY, J.G. Metabolismo de nucleotídeos de purina e pirimidina. In: DEVLIN, T.M. (ed), Manual de Bioquímica com correlações clínicas, 4nd edn. São Paulo, Edgard Blucher LTDA, pp. 408-436, 1998.

CRUZ, R., ALMAGUER-MELIAN, W., BERGADO – ROSADO, J.A. El glutatión em la función cognitiva y la neurodegeneración. Rev. Neurol. 36: 877-886, 2003.

CUSUMANO, F.J., PENNA, K.J., PANOSSIAN, G. Prevention of self-mutilation in patients with Lesch-Nyhan syndrome: review of literature. ASDC J. Dent. Child. 68: 175-178, 2001.

DASHEIFF, R.M. Benzodiazepinic treatment for Lesch-Nyhan syndrome? Dev. Med. Child. Neurol. 22:101-102, 1980.

DeANTONIO, I., TORRES-JIMENEZ, R., VERDÚ-PÉREZ, A., PRIOR de CASTRO, C., GARCIA-PUIG, J. Tratamiento del síndrome de Lesch-Nyhan. Rev. Neurol. 35: 877-883, 2002.

DE LEONIBUS, E., PASCUCCI, T., LOPEZ, S., OLIVERIO, A., AMALRIC, M., MELE, A. Spatial deficits in a mouse model of Parkinson disease. Psychopharmacology (Berl). 194(4):517-25, 2007

DELWING, D., GONCALVES, M.C., SARKIS, J.J., WYSE, A.T. L-NAME administration prevents the inhibition of nucleotide hydrolysis by rat blood serum subjected to hyperargininemia. Amino Acids. 29(3): 267-272, 2005.

DELWING, D., BAVARESCO, C.S., MONTEIRO, S.C., MATTÉ, C., NETTO, C.A., WYSE, A.T. alpha-Tocopherol and ascorbic acid prevent memory deficits provoked by chronic hyperprolinemia in rats. Behav Brain Res. 168(2): 185-189, 2006.

DELWING, D., DELWING, D., CHIARANI, F., KUREK, A.G., WYSE, A.T. Proline reduces brain cytochrome c oxidase: prevention by antioxidants. Int J Dev Neurosci. 25(1): 17-22, 2007a.

DELWING, D., DELWING, D., GONÇALVES, M.C., SARKIS, J.J., WYSE, A.T. NTPDase and 5'-nucleotidase activities of synaptosomes from hippocampus of rats subjected to hyperargininemia. Neurochem Res. 32(7): 1209-1216, 2007b.

DELWING, D., DELWING, D., SARKIS, J.J., WYSE, A.T. Proline induces alterations on nucleotide hydrolysis in synaptosomes from cerebral cortex of rats. Brain Res. 1149: 210-215, 2007c.

D'HOOGE, R. & DE DEYN, P.P. Applications of the Morris water maze in the study of learning and memory. Brain Res Brain Res Rev. 36(1): 60-90, 2001. Review.

DOBROTA, D., MATEJOVICOVA, M., KURELLA, E.G., BOLDYREV, A.A. Na/K-ATPase under oxidative stress: molecular mechanisms of injury. Cell Mol Neurobiol. 19(1):141-149, 1999.

DRÖGE, W. Free radicals in the physiological control of cell function. Physiol Rev. 82(1): 47-95, 2002. Review.

EDWARDS, N.L., RECKER, D., FOX, I.H. Overproduction of uric acid in hypoxanthine-guanine phosphoribosyltransferase deficiency: contribution by impaired purine salvage. J. Clin. Invest. 63: 922-930, 1979.

ENGIN, E. & TREIT, D. The role of hippocampus in anxiety: intracerebral infusion studies. Behav Pharmacol. 18(5-6):365-374, 2007.

EVELSON, P., TRAVACIO, M., REPETTO, M., ESCOBAR, J., LLESUY, S., LISSI, E.A. Evaluation of total reactive antioxidant potential (TRAP) of tissue homogenates and their cytosols. Arch. Biochem. Biophy. 388: 261-266, 2001.

FARDI, K., TOPOUZELIS, N., KOTSANOS, N. Lesch-Nyhan syndrome: a preventive approach to self-mutilation**.** Int. J. Paediatr. Dent. 13: 51-56, 2003.

FERNANDES, R. & SKIENA, S. MultiPrimer: a system for microarray PCR primer design. Methods Mol Biol. 402:305-314, 2007.

FLOYD, R.A. Antioxidants, oxidative stess, and degenerative neurological disorders. Proc. Soc. Exp. Biol. Med. 222 :236-245, 1999.

FRANZON, R., LAMERS, M.L., STEFANELLO, F.M., WANNMACHER, C.D., WAJNER, M., WYSE, A.T.S. Evidence that oxidative stress is involved in the inhibitory effect of proline on Na^+, K^+ - ATPase activity in synaptic plasma membrane of rat hippocampus. Int. J. Devl. Neurosci. 21:303-307, 2003.

FREI, B. On the role of vitamin C and other antioxidants in atherogenesis and vascular dysfunction. Proc. Soc. Exp. Biol. Med. 222: 196-204, 1999. Review.

GARCIA-ALLOZA, M., ZALDUA, N., DIEZ-ARIZA, M., MARCOS, B., LASHERAS, B., JAVIER GIL-BEA, F., RAMIREZ, M.J. Effect of selective cholinergic denervation on the serotonergic system: implications for learning and memory. J Neuropathol Exp Neurol. 65(11):1074-1081, 2006.

GENESTRA, M. Oxyl radicals, redox-sensitive signalling cascades and antioxidants. Cell Signal.19(9): 1807-1819, 2007.

GEYDE, A. Serotonin-gaba treatment is hypothesized for self-injury in Lesch-Nyhan syndrome. Med. Hyphot. 38: 325-328, 1992.

GIBBS, M.E., ANDREW, R.J., NG, K.T. Hemispheric lateralization of memory stages for discriminated avoidance learning in the chick. Behav Brain Res. 139(1-2): 157-165, 2003.

GIUGLIANI, R. Erros inatos do metabolismo: uma visão panorâmica. Pediatria Moderna. 1: 29-40, 1988.

GLOOR, S., ANTONICEK, H., SWEADNER, K.J., PAGLIUSI, S., FRANK, R., MOOS, M., SCHACHNER, M. The adhesion molecule on glial (AMOG) is a homologue of the beta subunit of the Na^+, K^+ - ATPase. J. Cell. Biol. 110 (1): 165-174, 1990.

GLYNN, I.M. Annual review prize lecture: all hands to the sodium pump. J. Physiol. 462 1-30, 1993.

GRAFIUS, M.A.., BOND, H.E., MILLAR, D.B. Acetylcholinesterase interaction with a lipoprotein matrix. Eur J Biochem. 22(3): 382-390, 1971.

GRISAR, T. Glial and neuronal Na^+,K^+-pump in epilepsy. Ann. Neurol**.** 16 (Suppl.): 128-134, 1984.

HABIBA, A., BALNCO. G., MERCER, R.W. Expression, activity and distribuition of Na^+,K^+-ATPase subunits during in vitro neuronal induction. Brain Res. 875(1-2): 1-13, 2000.

HALLIWELL, B. Free radicals, protein and DNA: oxidative damage versus redox regulation. Biochem. Soc. Trans. 24:1023-1027, 1996.

HALLIWELL, B. Oxidative stress and neurodegeneration: where are we now? J Neurochem. 97(6): 1634-1658, 2006. Review.

HARKNESS, M.A., McCREANOR, G.M., WATTS, R.W. Lesch-Nyhan syndrome and its pathogenesis: purine concentrations in plasma and urine with metabolite profiles in CSF. J. Inherit. Metab. Dis. 11: 239-252, 1988.

HASLER, U., CRAMBERT, G., HORISBERGER, J.D., GEERING, K. Structural and functional features of the transmembrane domain of the Na,K-ATPase beta subunit revealed by tryptophan scanning. J. Biol. Chem. 276(19):16356-16364, 2001.

HENDERSON, J.F. Possible functions of hypoxanthine-guanine phosphoribosyltransferase and their relation to the biochemical pathology of the Lesch-Nyhan syndrome. Fed. Proc. 27: 1075-1077, 1968.

HENDERSON, V.W., WATT, L., BUCKWALTER, J.G., Cognitive skills associated with estrogen replacement in women with Alzheimer's disease. Psychoneuroendocrinology 21: 421-430, 1996.

HIEBER, V., SIEGEL, G.J., FINK, D.J., BEATY, M.W., MATA, M. Differential distribution of (Na, K)-ATPase alpha isoforms in the central nervous system. Cell. Mol. Neurobiol. 11: 253-262, 1991.

HITCHLER, M.J. & DOMANN, F.E. An epigenetic perspective on the free radical theory of development. Free Radic Biol Med. 43(7):1023-1036, 2007.

HORTON, J.W. Free radicals and lipid peroxidation mediated injury in burn trauma: the role of antioxidant therapy. Toxicol. 189, 75 – 88, 2003.

IZQUIERDO, I. & MEDINA, J.H. Memory formation: the sequence of biochemical events in the hippocampus and its connection to activity in other brain structures. Neurobiol. Learn. Mem. 68(3): 285-316, 1997. Review.

IZQUIERDO, L.A., BARROS, D.M., VIANNA, M.R., COITINHO, A., DEDAVID E SILVA, T., CHOI, H., MOLETTA, B., MEDINA, J.H.,IZQUIERDO, I. Molecular pharmacological dissection of short- and long-term memory. Cell Mol Neurobiol. 22(3): 269-287, 2002.

IZQUIERDO, I., BEVILAQUA, L.R., ROSSATO, J.I., BONINI, J.S., DA SILVA, W.C., MEDINA, J.H., CAMMAROTA, M. The connection between the hippocampal and the striatal memory systems of the brain: a review of recent findings. Neurotox Res. 10(2):113-121, 2006.

JINNAH, H.A., GAGE, F.H., FRIEDMANN, T. Animal models of Lesch-Nyhan syndrome. Br ain. Res. Bull**.** 25: 467-475, 1990.

JINNAH, H.A. & FRIEDMANN, T. Lesch Nyhan disease and it variants. In: SCRIVER, C.R., BEAUDET, A.L., SLY, W.S., VALLE, D. (eds). The Metabolic and Molecular Basis of Inherited Disease, McGraw-Hill, New York, pp. 2537-2569, 2001.

JINNAH, H.A., HARRIS, J.C, NYHAN, W.L., O'NEILL, J.P. The spectrum of mutations causing HPRT deficiency: an update. Nucleosides Nucleotides Nucleic Acids. 23(8-9):1153-1160, 2004.

JINNAH, H.A., VISSER, J.E., HARRIS, J.C., VERDU, A., LAROVERE, L., CEBALLOS-PICOT, I., GONZALEZ-ALEGRE, P., NEYCHEV, V., TORRES, R.J., DULAC, O., DESGUERRE, I., SCHRETLEN, D.J., ROBEY, K.L., BARABAS, G., BLOEM, B.R., NYHAN, W., DE KREMER, R., EDDEY, G.E., PUIG, J.G., REICH, S.G.; LESCH-NYHAN DISEASE INTERNATIONAL STUDY GROUP. Delineation of the motor disorder of Lesch-Nyhan disease. Brain. 129(Pt 5): 1201-1217, 2006. Review

JORGENSEN, P.L., HAKANSSON, K.O., KARLISH, S.J.D. Structure and Mechanism of Na^+,K^+-ATPase: Functional sites and their interactions. Annu. Rev. Physiol. 65: 817-849, 2003.

KAPLAN, J.K. Biochemistry of Na^+,K^+-ATPase. Annu. Rev. Biochem. 71: 511-535, 2002.

KARL, T., PABST, R., VON HÖRSTEN, S. Behavioral phenotyping of mice in pharmacological and toxicological research. Exp Toxicol Pathol. 55(1): 69-83, 2003. Review.

KELLY, F.J. Use of antioxidants in the prevention and treatment of disease. J.C.C. 10: 21-23, 1998.

KISCH, S.J., FOX, I.H., KAPUR, B.M., LLOYD, K., HORNYKIEWICZ, O. Brain benzodiazepine receptor binding and purine concentration in Lesch-Nyhan syndrome. Brain. Res**.** 336: 117-123, 1985.

KITAMURA, N., IKEKITA, M., SATO, T., AKIMOTO, Y., HATANAKA, Y., KAWAKAMI, H., INOMATA, M., FURUKAWA, K. Mouse Na^+/K^+-ATPase beta1-subunit has a K+-dependent cell adhesion activity for beta-GlcNAc-terminating glycans. Proc. Natl. Acad. Sci. U. S. A. 102(8):2796-2801, 2005.

KOMOSZYNSKI, M. & WOJTCZAK, A. Apyrases (ATP diphosphohydrolases, EC 3.6.1.5): function and relationship to ATPases. Biochim Biophys Acta. 1310: 233-241, 1996.

KUMAR, A.R. & KURUP, P.A.. Inhibition of a membrane Na^+,K^+-ATPase activity: a common pathway in central nervous system disorder. J. Assoc. Physicians. India. 50: 400-406, 2002.

LANE, R.M., POTKIN, S.G., ENZ, A. Targeting acetylcholinesterase and butyrylcholinesterase in dementia. Int J Neuropsychopharmacol. 9(1): 101-124, 2006.

LASSITER, T.L., BARONE, S. JR., PADILLA, S. Ontogenetic differences in the regional and cellular acetylcholinesterase and butyrylcholinesterase activity in the rat brain. Brain Res Dev Brain Res. 105(1):109-123, 1998.

LEE, J.H., BERKOWITZ, R.J., CHOI, B.J. Oral self-mutilation in the Lesch-Nyhan syndrome**.** ASDC J. Dent. Child. 69: 66-69, 2002.

LEES, G.J., LEHMANN, A., SANDBERG, M., HAMBERGER, A. The neurotoxicity of ouabain, a sodium-potassium ATPase inhibition in the rat hippocampus. Neurosci. Lett. 120: 159-162, 1993.

LEHNINGER, A.L., NELSON, D.L., COX, M.M. Principles of Biochemistry, Worth Publishers, Inc. New York, 2nd Ed., 1995.

LESCH, M. & NYHAN, W.L. A familial disorder of uric acid metabolism and central nervous system function. Am J Med. 36: 561, 1964.

LIDBERG, L., ASBERG, M., SUNDQVIST-STENSMAN, U.B. 5-hydroxyindoliacetic acid levels in attempted suicides who have killed their children. Lancet 2: 928, 1984.

LINGREL, J.B. & KUNTZWEILER, T. Na^+,K^+-ATPase. J. Biochem. Chem. 269: 196599-196662, 1994.

LOEFFLER, D.A., CAMP, D.M., JUNEAU, P.L., HAREL, E., LEWITT, P.A. Purine-induced alterations of dopamine metabolism in rat pheochromocytoma PC12 cells. Brain. Res. Bull. 52: 553-558, 2000.

LÓPEZ, O.L. & BECHER, J.T. Tratamiento de la enfermedad de Alzheimer. Rev. Neurol. 35: 850-859, 2002.

LOPEZ, L.B., QUINTAS, E.M., NOEL, F. Influence of development on Na^+/K^+ ATPase expression: isoform-and tissue-depedency. Com. Biochem. Physiol. A. 131: 323-333, 2002.

LOPINA, A.D. Interaction of Na,K-ATPase catalytic subunit with cellular proteins and other endogenous regulators. Biochemistry (Mosc). 66(10): 1122-31, 2001.

LUNKES, G.I., LUNKES, D.S., MORSCH, V.M., MAZZANTI, C.M., MORSCH, A.L., MIRON, V.R., SCHETINGER, M.R. NTPDase and 5'-nucleotidase activities in rats with alloxan-induced diabetes. Diabetes Res Clin Pract. 65(1):1-6, 2004.

LYHAN, W.L. The recognition of Lesch-Nyhan syndrome as an inborn error of purine metabolism. J. Inher. Metab. Dis. 20: 171-178, 1997.

MA, M.H.Y., STACEY, N.C., CONNOLLY, G.P. Hypoxanthine impairs morphogenesis and enhances proliferation of a neuroblastoma model of Lesch Nyhan syndrome. J. Neurosci. Res. 63: 500-508, 2001.

MAHON, S., DENIAU, J.M., CHARPIER, S. Corticostriatal plasticity: life after the depression. Trends Neurosci. 27(8): 460-467, 2004. Review.

MARKLUND, S.L. Product of extracellular-superoxide dismutase catalysis. FEBS Lett. 184: 237-239, 1985.

MARTEL, G., BLANCHARD, J., MONS, N., GASTAMBIDE, F., MICHEAU, J., GUILLOU, J.L. Dynamic interplays between memory systems depend on practice: The hippocampus is not always the first to provide solution. Neuroscience. 150(4):743-753, 2007.

MATEOS, F.A., PUIG, J.G., RAMOS, T.H., JIMÉNEZ, M.L., ROMERA, N.M., GONZALEZ, A.G. Prenatal diagnosis of Lesch-Nyhan syndrome by purine analysis of amniotic fluid and cordocentesis. Adv. Exp. Med. Biol. 309B: 47-50, 1991.

MAULIK, D., QAYYUM, I., POWELL, S.R., KARANTZA, M., MISHRA, O.P., DELIVORIA-PAPADOPOULOS, M. Post-hypoxic magnesium decreases nuclear oxidative damage in the fetal guinea pig brain. Brain Res. 890(1):130-136, 2001.

MATTÉ, C., SCHERER, E.B., STEFANELLO, F.M., BARSCHAK, A.G., VARGAS, C.R., NETTO, C.A, WYSE, A.T. Concurrent folate treatment prevents Na(+),K(+)-ATPase activity inhibition and memory impairments caused by chronic hyperhomocysteinemia during rat development. Int. J. Dev. Neurosci. 25(8): 545-552, 2007.

MATTHEWS, W.S., SOLAN, A., BARABAS, G. Cognitive functioning in Lesch-Nyhan syndrome. Dev Med Child Neurol. 37(8):715-722, 1995.

MEDINA, J.H., SCHRÖDER, N., IZQUIERDO, I. Two different properties of short- and long-term memory. Behav Brain Res. 103(1): 119-121, 1999.

MICHIELS, C., RAES, M., TOUSSAINT, O., REMACLE, J., Importance of Se-glutathione peroxidase, catalase, and Cu/Zn-SOD for cell survival against oxidative stress. Free Rad. Biol. Med. 17: 235-248, 1994.

MONTEIRO, S.C., MATTOS, C.B., SCHERER, E.B., WYSE, A.T. Supplementation with vitamins E plus C or soy isoflavones in ovariectomized rats: effect on the activities of $Na(^{+})$, K ($^{+}$)-ATPase and cholinesterases. Metab. Brain Dis. 22: 156-171, 2007.

MORENO, S., MUGNAINI, E., CERÙ, M.P. Immunocytochemical localization of catalase in the central nervous system of the rat. J Histochem Cytochem. 43(12):1253-1267, 1995.

MORIWAKI, Y., YAMAMOTO, T., HIGASHINO, K. Enzymes involved in purine metabolism – a review of histochemical localization and functional implications. Histol. Histopathol. 14: 1321-1340, 1999.

NANITSOS, E.K., NGUYEN, K.T., ST'ASTNÝ, F., BALCAR, V.J. Glutamatergic hypothesis of schizophrenia: involvement of Na+/K+-dependent glutamate transport. J. Biomed. Sci. 12(6): 975-984, 2005.

NEDELJKOVIC, N., BJELOBABA, I., SUBASIC, S., LAVRNJA, I., PEKOVIC, S., STOJKOV, D., VJESTICA, A., RAKIC, L., STOJILJKOVIC, M. Up-regulation of ectonucleotidase activity after cortical stab injury in rats. Cell Biol Int. 30: 541-546, 2006.

NETTO, C.A., HODGES, H., SINDEN, J.D., LEPEILLET, E., KERSHAW, T., SOWINSKI, P., MELDRUM, B.S., GRAY, J.A. Foetal grafts from hippocampal regio superior alleviate ischaemic-induced behavioural deficits. Behav Brain Res. 58(1-2): 107-112, 1993.

NEYCHEV, V.K. & MITEV, V.I. The biochemical basis of the neurobehavioral abnormalities in the Lesch-Nyhan syndrome: a hypothesis. Med Hypotheses. 63: 131-134, 2004.

NYHAN, W.L. The Lesch-Nyhan syndrome. Annu. Rev. Med**.** 24:41-60, 1973.

NYHAN, W.L. The Lesch-Nyhan syndrome. Dev. Med. Chil. Neurol. 20: 376-380, 1978.

OHKAWA, H., OHISHI, N., YAGI, K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal. Biochem. 95: 351-358, 1979.

OLSON, L. & HOULIHAN, D. A review of behavioral treatments used for Lesch-Nyhan syndrome. Behav. Modific. 24:202-222, 2000.

OZDEN, S. & ISENMANN, S. Neuroprotective properties of different anesthetics on axotomized rat retinal ganglion cells in vivo. J Neurotrauma 21 :73-82, 2004.

PAXINOS, G. & WATSON, C. The rat brain in stereotaxic coordinates. Academic Press, London, 1986.

PALMOUR, R.M., HESHKA, T.W., ERVIN, F.R. Hypoxanthine accumulation and dopamine depletion in Lesch-Nyhan disease. Adv. Exp. Med. Biol**.** 253: 165-172, 1989.

PESI, R., MICHELI, V., JACOMELLI, G., PERUZZI, L., CAMICI, M., GARCIA-GIL, M., ALLEGRINI, S., TOZZI, M.G. Cytosolic 5'-nucleotidase hyperactivity in erythrocytes of Lesch-Nyhan syndrome patients. Neuroreport. 9: 1827-1831, 2000.

PRIOR, C., TORRES, R.J., PUIG, J.G. Hypoxanthine effect on equilibrative and concentrative adenosine transport in human lymphocytes: implications in the phatogenesis of Lesch-Nyhan syndrome. Nucleosides Nucleotides Nucleic Acids. 25(9-11): 1065-1069, 2006.

PRIOR, C., TORRES, R.J., PUIG, J.G. Hypoxanthine decreases equilibrative type of adenosine transport in lymphocytes from Lesch-Nyhan patients. Eur J Clin Invest. 37(11): 905-911, 2007.

PUIG, J.G., JIMENEZ, M.L., MATEOS, F.A., FOX, I.H. Adenine nucleotide turnover in hypoxanthine-guanine phosphoribosyl-transferase: evidence for an increased contribution of purine biosyntheses de novo. Metabolism. 38: 410-418, 1989.

PUIG, J.G., & MATEOS, F.A. The biochemical basis of HGPRT deficiency. In: Gresser, U. (ed). Molecular genetics, biochemistry and clinical aspects of inherited disorders of purine and pyrimidine metabolism. Springer-Verlag, New York. pp. 12-26, 1993.

REZNICK, A.Z., & PACKER, L. Free radicals and antioxidants in muscular neurological diseases and disorders. In: Poli, G., Albano, E., and Dianzani, M.U. (eds), Free Radicals: from Basic Science to Medicine, Birkhäuser Verlag, Basel. pp. 425-437, 1993.

RIBEIRO, J.A., LOBO, M.G., SEBASTIÃO, A.M. Endogenous adenosine modulation of 22Na uptake by rat brain synaptosomes. Neurochem Res. 28: 1591-1595, 2003.

ROBEY, K.L., RECK, J.F., GIACOMINI, K.D., BARABAS, G., EDDEY, G.E. Modes and patterns of self-mutilation in persons with Lesch-Nyhan disease. Dev. Med. Child. Neurol. 45: 167-171, 2003.

ROSSATO, J.I., ZINN, C.G., FURINI, C., BEVILAQUA, L.R., MEDINA, J.H., CAMMAROTA, M., IZQUIERDO, I. A link between the hippocampal and the striatal memory systems of the brain. An. Acad. Bras. Cienc. 78(3):515-523, 2006

ROSSITER, B.J.F. & CASKEY, C.T. Hypoxanthine-guanine phosphoribosyltransferase deficiency: Lesch-Nyhan syndrome and gout. In: SCRIVER, C.R., BEAUDET, A.L., SLY, W.S., VALLE, D. (eds), The Molecular Bases of Inherited Disease, McGraw-Hill, New York, pp. 1679-1706, 1995.

SAITO, Y. & TAKASHIMA, S. Neurotransmitter changes in the pathophysiology of Lesch-Nyhan syndrome. Brain Dev. 22: S122-131, 2000.

SALEM, N. JR. & TRAMS, E.G. Ecto-5'-nucleotidase regeneration after chemical modification of the plasma membrane. Neurochem Res. 8(1):39-49, 1983.

SCHRETLEN, D.J., HARRIS, J.C., PARK, K.S., JINNAH, H.A., DEL POZO, N.O. Neurocognitive functioning in Lesch-Nyhan disease and partial hypoxanthine-guanine phosphoribosyltransferase deficiency. J Int Neuropsychol Soc. 7(7):805-812, 2001.

SCHRETLEN, D.J., WARD, J., MEYER, S.M., YUN, J., PUIG, J.G., NYHAN, W.L., JINNAH, H.A., HARRIS, J.C. Behavioral aspects of Lesch-Nyhan disease and its variants. Dev Med Child Neurol. 47(10): 673-677, 2005.

SCRIVER, C.R., BEAUDET, A.L., SLY, W.S. and VALLE, D. The metabolic and molecular bases of inherited disease. McGraw-Hill, Inc., New York, 8th Ed., 2001.

SEEGMILLER, J.E, ROSENBLOOM, F.M., KELLEY, W.N. Enzyme defect associated with sex-linked human neurological disorder and excessive purine synthesis. Science. 155: 1682, 1967.

SEEGMILLER, J.E. Inherited deficiency of hypoxanthine-guanine phosphoribosyltransferase in X-linked. Adv. Hum. Genet. 6: 75-163, 1976.

SERRANO, F. & KLANN, E. Reactive oxygen species and synaptic plasticity in the aging hippocampus. Ageing Res. Rev. 3: 431- 443, 2004.

SHERRY, J.M. & CROWE, S.F. Ouabain does not impair reconsolidation following a reminder of passive avoidance learning in the day-old chick. Neurosci Lett. 423(2):123-127, 2007.

SHIRLEY, T.L., LEWERS, J.C., EGAMI, K., MAJUMDAR, A., KELLY, M., CEBALLOS-PICOT, I., SEIDMAN, M.M., JINNAH, H.A. A human neuronal tissue culture model for Lesch-Nyhan disease. J Neurochem. 101(3): 841-853, 2007.

SIEMS, W.G., HAPNER, S.J., VAN KUIJK, F.J. 4 – hydroxynonenal inhibits Na^+,K^+-ATPase. Free. Rad. Biol. Med. 20: 215-223, 1996.

SIMINTZI, I., SCHULPIS, K. H., ANGELOGIANNI, P., LIAPI, C., TSAKIRIS, S. L-Cysteine and glutathione restore the reduction of rat hippocampal Na^+, K^+-ATPase activity induced by aspartame metabolites. Toxicology 237(1-3): 177-183, 2007.

SKOU, J.C. & ESMANN, M. The Na^+,K^+-ATPase. J. Bioenerg. Biomembr., 24:249-261, 1992.

STEFANELLO, F.M., SCHERER, E.B., KUREK, A.G., MATTOS, C.B., WYSE, A.T. Effect of hypermethioninemia on some parameters of oxidative stress and on Na(+),K (+)-ATPase activity in hippocampus of rats. Metab. Brain Dis. 22(2):172-182, 2007.

STRECK, E.L., ZUGNO, A.I., TAGLIARI, B., FRANZON, R., WANNMACHER, C.M.D., WAJNER, M., WYSE, A.T.S. Inhibition of rat brain Na^+,K^+-ATPase activity induced by homocisteine is probably mediated by oxidative stress. Neurochem. Res. 26: 195-200, 2001.

SWEADNER, K.J. The sodium pump: recent developments. In: KAPLAN, J.H., DE WEER, P. eds. Society of General Physiologists Series, Vol. 46 (Part I). Rockefeller University Press, New York, pp. 63-76, 1991.

SWEETMAN, L. Urinary and cerebrospinal fluid oxypurine levels and allopurinol metabolism in the Lesch-Nyhan syndrome. Fed. Proc. 27: 1055-1059, 1968.

THERIEN, A.G., GOLDSHLEGER, R., KARLISH, S.J.D., BLOSTEIN, R. Tissue-specific distribuition and modulatory role of the γ subunit of the Na^+,K^+-ATPase. J. Biol. Chem. 272: 32623-32634, 1997.

TICKU, M.K. & BURCH, T. Purine inhibition of [^{3}H]- gamma -aminobutyric acid receptor binding to rat brain membranes. Biochem. Pharmacol. 29: 1217-1220, 1980.

TORRES, R.J., DEANTONIO, I., PRIOR, C., PUIG, J.G. Adenosine transport in HPRT deficient lymphocytes from Lesch-Nyhan disease patients. Nucleosides Nucleotides Nucleic Acids. 23: 1193-1196, 2004.

TORRES-JIMENEZ, R., MATEOS-ANTÓN, F., ARCAS-MARTINEZ, J., PASCUAL-CASTROVIEJO, I., GARCIA-PUIG, J. Fisiopatologia de las manifestaciones neurológicas em la deficiência de hipoxantina-guanina fosforribosiltransferasa. Rev. Neurol. 27: 1050-1054, 1998.

TRAVACIO, M. & LLESUY, S. Antioxidant enzymes and their modification under oxidative stress conditions. Free Radic. Res. Latin Am. 48: 9–13, 1996.

TSENOV G, MÁTÉFFYOVÁ A, MARES P, OTÁHAL J, KUBOVÁ H. Intrahippocampal injection of endothelin-1: a new model of ischemia-induced seizures in immature rats. Epilepsia 48 Suppl 5: 7-13, 2007.

VALKO, M., LEIBFRITZ, D., MONCOL, J., CRONIN, M.T., MAZUR, M., TELSER, J. Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol. 39(1): 44-84, 2007.

VASILETS, L.A. & SCHWARZ, W. Structure-function relationships of cation binding in the Na^+,K^+-ATPase. Biochim. Biophys. Acta. 1154: 201-222, 1993.

VIETTA, M., FRASSETTO, S.S., BATTASTINI, A.M., BELLO-KLEIN, A., MOREIRA, C., DIAS, R.D., SARKIS, J.J. Sensitivity of ATPase-ADPase activities from synaptic plasma membranes of rat forebrain to lipid peroxidation in vitro and the protective effect of vitamin E. Neurochem Res. 21: 299-304, 1996.

VILLA, R.F., GORINI, A., HOYER, S. ATPases of synaptic plasma membranes from hippocampus after ischemia and recovery during ageing. Neurochem. Res. 27(9):861-870, 2002.

VISSER, J.E., BAR, P.R., JINNAH, H.A. Lesch-Nyhan disease and the basal ganglia. Brain Res. Bull. 32: 449-475, 2000.

VISSER, J.E., SMITH, D.W., MOY, S.S., BREESE, G., FRIEDMANN, T., ROTHSTEIN, J.D., JINNAH, H.A. Oxidative stress and dopamine deficiency in genetic mouse model of Lesch-Nyhan disease. Brain. Res. Dev. Brain. Res. 28: 127-139, 2002.

VUADEN, F.C., DE PAULA COGNATO, G., BONORINO, C., BOGO, M.R., DE FREITAS SARKIS, J.J., BONAN, C.D. Lipopolysaccharide alters nucleotidase activities from lymphocytes and serum of rats. Life Sci. 80: 1784-1791, 2007.

WARNER, D.S., SHENG, H., BATINIĆ-HABERLE, I. Oxidants, antioxidants and the ischemic brain. J Exp Biol. 207(Pt 18): 3221-3231, 2004. Review.

WENDEL, A. Glutathione peroxidase. Methods Enzymol.77: 325-333, 1981.

WESTERNINK, B.H.C., DAMSMA, G., VRIES, J.B. Efeect of ouabain applied by intrastriatal microdialysis on the in vivo release of dopamine, acetylcholine, and amino acids in the brain of conscious rats. J. Neurochem. 52: 705-712, 1990.

WHITE, N.M. & MCDONALD, R.J. Multiple parallel memory systems in the brain of the rat. Neurobiol. Learn. Mem. 77: 125 – 184, 2002.

WYSE, A.T.S, STRECK, E.L., WORM, P., WAJNER, M., RITTER, F., NETTO, C.A. Preconditioning prevents the inhibition of Na^+,K^+-ATPase activity after brain ischemia. Neurochem. Res. 25: 969-973, 2000.

WYSE, A.T.S, ZUGNO, A.I., STRECK, E.L., MATTE, C., CALCAGNOTTO, T., WANNMACHER, C.D., WAJNER, M. Inhibition of Na^+,K^+-ATPase activity in hippocampus of rats subjected to acute administration of homocysteine is prevented by vitamins E and C treatment. Neurochem. Res. 27: 1685-1689, 2002.

WYSE, A.T., BAVARESCO, C.S., REIS, E.A., ZUGNO, A.I., TAGLIARI, B., CALCAGNOTTO, T., NETTO, C.A. Training in inhibitory avoidance causes a reduction of Na+,K+-ATPase activity in rat hippocampus. Physiol Behav. 80(4):475-479, 2004.

XIAO, A.Y., WANG, X.Q., YANG, A., YU, S.P (b). Sligth impairment of Na^+,K^+-ATPase synergistically aggravates ceramide and beta-amyloid induced apoptosis in cortical neurons. Brain. Res. 955 (1-2): 253-259, 2002.

ZIMMERMANN, H. Ectonucleotidases in the nervous system. Novartis Found Symp. 276: 113-128, 2006.

ZHU, C.B., STEINER, J.A., MUNN, J.L., DAWS, L.C., HEWLETT, W.A., BLAKELY R.D. Rapid stimulation of presynaptic serotonin transport by a3 adenosine receptors. J. Pharmacol. Exp. Ther. 322: 332-340, 2007

ZUGNO, A.I., SCHERER, E.B., MATTOS, C., RIBEIRO, C.A., WANNMACHER, C.M., WAJNER. M., WYSE, A.T. Evidence that the inhibitory effects of guanidinoacetate on the activities of the respiratory chain, Na+,K+-ATPase and creatine kinase can be differentially prevented by taurine and vitamins E and C administration in rat striatum in vivo. Biochim Biophys Acta. 1772: 563-569, 2007.