UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS:

BIOQUÍMICA

EFEITOS DA HOMOCISTEÍNA SOBRE PARÂMETROS BIOQUÍMICOS E ESTRUTURAIS DO CITOESQUELETO DE CÉLULAS NEURAIS DE RATOS

Samanta Oliveira Loureiro

Porto Alegre

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EFEITOS DA HOMOCISTEÍNA SOBRE PARÂMETROS BIOQUÍMICOS E ESTRUTURAIS DO CITOESQUELETO DE CÉLULAS NEURAIS DE RATOS

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Tese apresentada ao Curso de Pós-Graduação em Ciências Biológicas: Bioquímica, como requisito parcial à obtenção do grau de Doutror em Bioquímica.

Porto Alegre

2009

Dedicatória

Dedico esse trabalho aos meus pais que são

os meus maiores incentivadores e

responsáveis por tudo que sou.

O começo de todas as ciências é estranhar

que as coisas sejam como são.

Aristóteles

Nada existe de grandioso sem Paixão.

Georg Wilhelm Friedrich Hegel

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PARTE I

RESUMO

A homocistinúria (HHCY) é uma desordem metabólica causada algum tipo de deficiência no metabolismo da metionina, folato ou vitamina B12, resultando no acúmulo tecidual de homocisteína (Hcy) e de metionina. Os pacientes afetados por essa doença apresentam principalmente retardo mental, isquemia cerebral, convulsões e aterosclerose. Vários mecanismos têm sido propostos para explicar a relação entre HHCY e desordens no sistema nervoso, entre eles podemos destacar mecanismos glutamatérgicos, mobilização de Ca^{+2} e envolvimento de espécies reativas de oxigênio (ROS). Considerando que o citoesqueleto é um importante alvo para a sinalização celular em inúmeras doenças neurodegenerativas, nosso estudo investigou os possíveis efeitos tóxicos da Hcy sobre alguns parâmetros bioquímicos do citoesqueleto neural. Ratos submetidos a um tratamento crônico com Hcy apresentam uma marcada seletividade na alteração da expressão gênica das subunidades dos filamentos intermediários (FIs) estudados, tanto no extrato tecidual total como na fração citoesquelética de hipocampo e córtex cerebral, refletindo uma maior susceptibilidade do hipocampo nessas alterações. Estudos *in vitro* mostraram que a Hcy 100 e 500 µM, relacionadas a homocistinuria (HHCY) moderada e grave respectivamente, são capazes de causar hiper (Hcy 100 µM) ou hipofosforilação (Hcy 500 µM) das subunidades dos neurofilamentos e da proteina glial fibrilar ácida, FIs do citoesqueleto de neurônios e astrócitos respectivamente. Estes efeitos são dependentes da idade dos ratos e da estrutura cerebral, manifestando-se em hipocampo de ratos de 17 dias de idade. Resultados em fatias de hipocampo mostraram que a ação das duas concentrações de Hcy é mediada por mecanismos dependentes do influxo de Ca^{2+} por receptores NMDA e por canais de Ca^{2+} dependentes de voltagem, assim como da liberação de Ca^{2+} dos estoques intracelulares, enfatizando a alta suscetibilidade e complexidade das vias de sinalização ativadas por Ca^{2+} no

hipocampo. Além disso, estudos morfológicos em astrócitos e células de glioma C6 mostraram que células glias em cultura também são alvo para as ações da Hcy, reorganizando seu citoesqueleto e alterando o sistema fosforilante associado ao mesmo através de mecanismos envolvendo excitotoxicidade, estresse oxidativo e mecanismos glutamatérgicos. Estes estudos mostram que a integridade estrutural do citoesqueleto é da maior importância para o funcionamento neuronal e qualquer distúrbio na dinâmica desta estrutura poderia ativar processos de reparação plástica manifestados como alterações na expressão, localização e metabolismo das proteínas do citoesqueleto. No entanto, os mecanismos através dos quais o desequilíbrio do citoesqueleto é capaz de induzir a disfunção neural ainda não foram esclarecidos e a exata dimensão destas alterações precisa ser determinada.

ABSTRACT

The homocystinuria (HHCY) is a metabolic disorder caused by deficiency in the metabolism of methionine, vitamin B12 or folate, which leads to tissue accumulation of homocysteine (Hcy) and methionine. Homocystinuric patients usually present mental retardation, cerebral ischemia, seizures, and atherosclerosis. Several mechanisms have been proposed to explain the relationship between HHCY and nervous system disorders, among them we can highlight glutamatergic mechanisms, mobilization of Ca^{+2} and involvement of reactive oxygen species (ROS). Considering that the cytoskeleton is an important target for cellular signaling in many neurodegenerative diseases, our study investigated the possible toxic effects of Hcy on some biochemical parameters of the neural cytoskeleton. Initially, we demonstrated that rats subjected to chronic model of Hcy showed a marked selectivity in alterations of gene expression, total immunocontent and cytoskeletal fraction of subunits of

intermediate filaments (IFs) studied, reflecting a greater susceptibility of the hippocampus in these changes.

In vitro studies showed that 100 and 500 μM Hcy associated with moderate and severe HHCY respectively, was able to induced hyperphosphorylation (Hcy 100 μM) and hypophosphorylation (Hcy 500 μM) of neurofilaments subunits and glial fibrillary acidic protein, IFs of neuronal and astrocytic cytoskeleton. These effects are dependentents of age and cerebral structure of rats: hippocampus of 17 day old rats are sensible. Results in slices of hippocampus showed that the action of Hcy is mediated by mechanisms dependent of influx of Ca^{2+} by NMDA receptors and Ca^{2+} channels voltage-dependent and release of Ca^{2+} from intracellular stores, emphasizing the high susceptibility and complexity of signaling pathways activated by Ca^{2+} in the hippocampus. In addition, glial cells were also target for the actions of Hcy, reorganizing their cytoskeleton and changing the phosphorylation system associated to cytoskeleton through mechanisms involving excitotoxicity, oxidative stress and glutamatergic mechanisms. The cytoskeleton may represent a target to HHCY and your dysfunction can have an important role in neurodegeneration characteristic of the disease. However, the mechanisms by which disruption of the cytoskeleton proteins is able to induce neural dysfunction have not yet been clarified and the exact extent of these changes need to be determined.

LISTA DE ABREVIATURAS

AMP	Adenosina 5'-monofosfato
AMPc	AMP cíclico
AMPA	(*R* ,*S*)-α-amino-3-hidroxi-5-metil-4-isoxazolepropionico ácido
Ca^{+2}	Cálcio
CBS	Cistationina β-sintetase
FIs	Filamentos Intermediários
GFAP	Proteína glial fibrilar ácida
GTP	Guanosina trifosfato
Hcy	Homocisteína
HHCY	Hiper-homocisteinemia
iGluRs	Receptores glutamatérgicos ionotrópicos
JNK	Proteína quinase Jun-amino-terminal
KA	Cainato
LTD	Depressão de longa duração
LTP	Potencialização de longa duração
MAPK	Proteína quinase ativada por mitógeno
MFs	Microfilamentos
MTs	Microtúbulos
mGluRs	Receptores glutamatérgicos metabotrópicos
NFs	Neurofilamentos
NF-L	Neurofilamento de baixo peso molecular
NF-M	Neurofilamento de médio peso molecular
NF-H	Neurofilamento de alto peso molecular

NMDA	N-metil-D-aspartato
PI3K	Fosfatidilinositol 3-quinase
PKA	Proteína quinase A
PKC	Proteína quinase C
PKCamII	Proteína quinase dependente de cálcio e calmodulina II
PLC	Fosfolipase c
PP1	Proteína fosfatase 1
PP2A	Proteína fosfatase 2A
PP2B	Proteína fosfatase 2B
ROS	Espécies reativas de oxigênio
SAH	S-adenosil homocisteína
SAM	S-adenosil metionina
SNC	Sistema Nervoso Central
VDCC	Canais de Ca^{+2} dependentes de voltagem

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1. INTRODUÇÃO

1.1 HOMOCISTEÍNA

1.1.1 Metabolismo da Homocisteína

A homocisteína (Hcy) é um aminoácido sulfurado, não essencial, derivado do metabolismo da metionina. O catabolismo da Hcy depende de folato, vitamina B12 e vitamina B6 (Obeid et al., 2004). A metabolização da Hcy pode seguir duas vias: a via da remetilação, a qual envolve a síntese de metionina, onde folato e vitamina B12 são co-fatores, ou a via da transsulfuração, a qual envolve a degradação de Hcy e possui a vitamina B6 como co-fator (Selhub, 1999).

Aproximadamente, metade da Hcy gerada é remetilada a metionina, utilizando-se 5-metiltetrahidrofolato (5-CH_3-THF) ou betaína como doador de grupos metil. Algum desequilíbrio nesse processo pode causar uma diminuição de S-adenosil metionina (SAM) e um aumento de S-adenosil homocisteína (SAH), um potente inibidor de metil transferases. Dentre as diversas funções biológicas exercidas por grupos metil no Sistema Nervoso Central (SNC), pode-se destacar a síntese e metabolismo de vários neurotransmissores bem como a manutenção da metilação do DNA (Chadwick et al., 2000; Gharib et al., 1983; Mattson e Shea, 2003; McKeever et al., 1991).

A rota da transulfuração da Hcy é considerada um importante suprimento de glutationa para o fígado, no entanto, esta rota é pouco conhecida no cérebro. Cistationina β-sintetase (CBS) (EC 4.2.1.22) e cistationase (EC 4.4.1.1) catalisam a transulfuração da Hcy em cisteína (Cys), precursora da glutationa. A enzima CBS tem sido detectada em cérebro humano (Ichinohe et al., 2005), no entanto, estudos a respeito da cistationase em cérebro ainda não são conclusivos (Awata et al., 1995; Griffiths e Tudball, 1976; Heinonen, 1973).

Além disso, há evidências que mostram grandes variações regionais na atividade da cistationase no cérebro (Awata et al., 1995; Griffiths e Tudball, 1976). Em geral, os dados disponíveis não são suficientes para definir o papel da via de transulfuração na produção de cisteína necessária para a síntese de glutationa no cérebro (Kranich et al., 1998; Scott et al., 1994; Shanker et al., 2001).

Estudos indicam que o transporte de Hcy do sangue para o cérebro, além de simples difusão, pode ser feito via um receptor específico saturável (Grieve et al., 1992; Griffiths et al., 1992; Zeise et al., 1988). Além disso, células neuronais humanas são capazes de produzir Hcy em condições normais (Ho et al., 2003).

Concentrações de Hcy no cérebro e no fluido cérebro-espinhal são elevadas em diversas doenças neurodegenerativas (Eto et al., 2002; Isobe et al., 2005; Regland et al., 2004; Regland et al., 1997; Yanai et al., 1983). Recentes evidências também demonstraram, em um modelo de hiper-homocisteinemia (HHCY), um comprometimento da integridade da barreira sangue-cérebro (Kamath et al., 2006).

1.1.2 Hiper-homocisteinemia

Os níveis plasmáticos de Hcy podem variar consideravelmente entre os indivíduos devido a fatores genéticos, dietéticos e ambientais, drogas e função renal (Dierkes e Westphal, 2005; Klerk et al., 2002; Kruger et al., 2003; Mudd et al., 1985; Selhub et al., 1993; van Guldener e Stehouwer, 2005; Watkins et al., 2002).

Clinicamente, é usual considerar que em adultos as concentrações plasmáticas de Hcy podem variar de 5 a 10 μmol/L e que níveis plasmáticos de 12 a 15 μmol/L tem sido associados com risco elevado de doenças cardiovasculares e neurodegenerativas (Herrmann, 2001; Mattson e Shea, 2003).

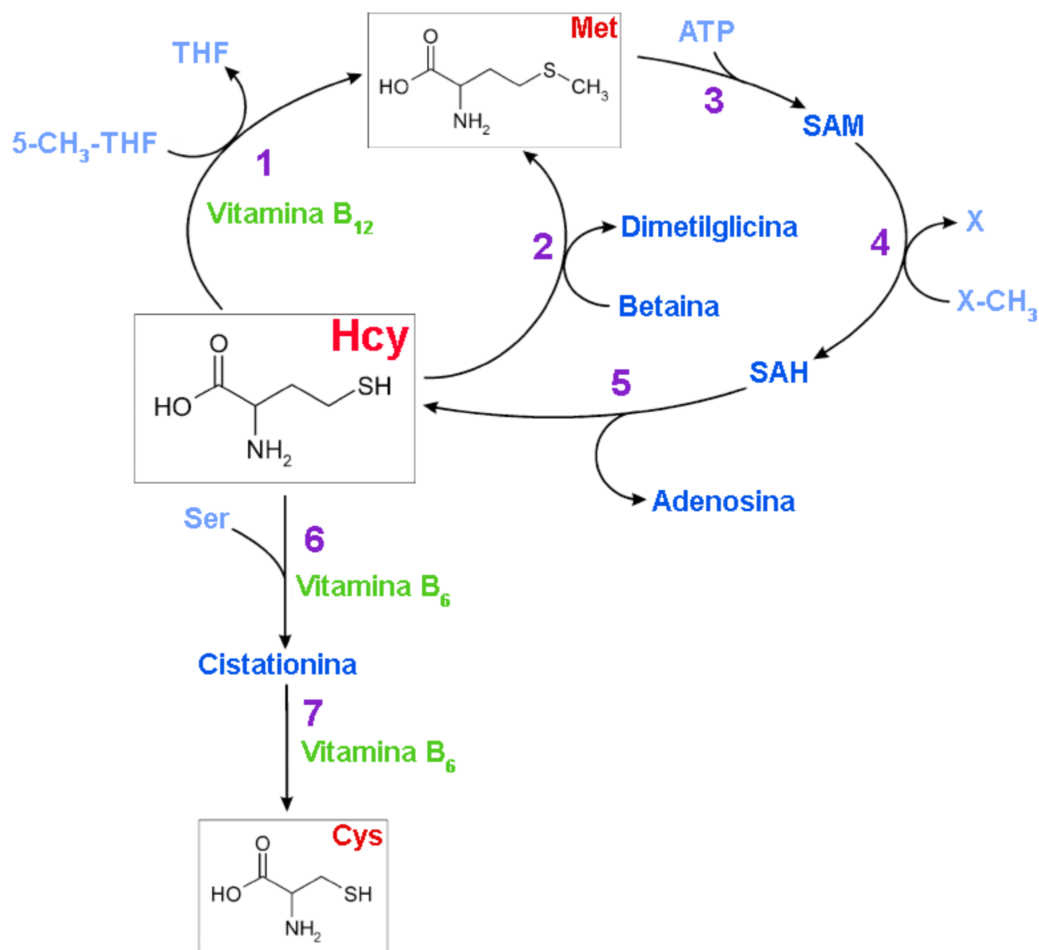

Figura 1. Metabolismo da Hcy. 1) metionina sintase; 2) betaina-homocisteina metiltransferase; 3) metionina adenosiltransferase; 4) metiltranferase; 5) SAH hydrolase; 6) cistationina β-sintase; 7) cistationina γ-liase; Hcy: homocisteína; Met: metionina; Cys: cisteína; SAM: S-adenosil Metinina; SAH: S-adenosil homocisteína; 5-CH_3-THF: 5-metiltetrahidrofolato; THF: tetrahidrofolato.

HHCY é definida como uma elevação na concentração plasmática de Hcy (Mudd et al., 2000). A HHCY grave (concentrações plasmáticas acima de 100 μmol/L) é classicamente causada por raros fatores genéticos no metabolismo da metionina, folato ou vitamina B12 (Dierkes e Westphal, 2005; Mudd et al., 1985; Tonetti et al., 2003; van Guldener e Stehouwer, 2005; Watkins et al., 2002), mas também pode ocorrer em indivíduos com severa deficiência de vitamina B12, atribuída à anemia perniciosa (Limal et al., 2006). A HHCY

moderada (concentrações plasmáticas de 10-100 µmol/L) pode ser causada por doenças renais, deficiências de folato ou vitamina B12, ou por um defeito genético no gene da metileno tetrahidrofolato redutase (Mudd et al., 2000).

1.1.3 Homocisteína e Doenças Neurodegenerativas

Uma ligação entre Hcy e desordens no sistema nervoso foi primeiramente descrita em pacientes com severa deficiência na CBS (Grieco, 1977; Mudd et al., 1985). Retardo mental, atrofia cerebral e convulsões foram relatados nesses pacientes (Grieco, 1977; Mudd et al., 1985; Sachdev et al., 2002; van den Berg et al., 1995). Posteriormente, também se observou uma relação entre Hcy e doenças relacionadas ao envelhecimento, incluindo declínio cognitivo (Budge et al., 2000; Kado et al., 2005), doenças cerebrovasculares e acidente vascular cerebral (Bostom et al., 1999; Yoo et al., 1998), demência vascular (Hogervorst et al., 2002) e doença de Alzheimer (Bosco et al., 2004; Seshadri et al., 2002). Elevação nos níveis de Hcy foi também associada com outras desordens do SNC, como a depressão (Bell et al., 1992) e a esquizofrenia (Applebaum et al., 2004; Levine et al., 2002).

1.1.4 Principais mecanismos envolvidos nos efeitos neurotóxicos da Hcy

Vários mecanismos têm sido propostos para explicar a relação entre HHCY e neurodegeneração (Dayal et al., 2004; Maler et al., 2003), dentre eles, podemos destacar três possibilidades hipotéticas (Bottiglieri, 1996; Mattson e Shea, 2003; Selhub, 1999).

A primeira é que a Hcy é diretamente neurotóxica (Mattson e Shea, 2003; Molloy, 2001). Essa possibilidade sugere que a Hcy é um composto endógeno, neurotóxico em concentrações supra fisiológicas. Dados da literatura demonstram que altas concentrações cerebrais de Hcy ou seus derivados oxidados podem alterar a neurotransmissão ou induzir excitotoxicidade em neurônios (Lipton e Rosenberg, 1994), particularmente aqueles

expressando receptores do tipo N-metil-D-aspartato (NMDA) (Lipton et al., 1997; Robert et al., 2004). A estimulação de receptores NMDA pode desencadear um aumento de cálcio citoplasmático e espécies reativas de oxigênio (ROS), seguido por neurotoxicidade e apoptose (Lafon-Cazal et al., 1993; Lipton et al., 1997). O envolvimento de receptores glutamatérgicos metabotrópicos (mGluR) também foi demonstrado (Lazarewicz et al., 2003; Zieminska e Lazarewicz, 2006; Zieminska et al., 2003). Além disso, a auto-oxidação da Hcy leva a formação de superóxido e peróxido de hidrogênio, os quais subseqüentemente alteram a função neuronal e predispõem o tecido neuronal afetado a doenças neurodegenerativas (White et al., 2001). Além disso, Hcy inibe a expressão de enzimas antioxidantes, como a glutationa peroxidase, potencializando os efeitos tóxicos de ROS (Upchurch et al., 1997).

A segunda possibilidade postula que HHCY ocasiona uma alteração no metabolismo de grupos metila, o qual se estende ao cérebro. Um acúmulo de Hcy pode aumentar SAH, o qual é um potente inibidor de muitas reações de metilação, essenciais para a função neurológica (Chen et al., 2001; Choumenkovitch et al., 2002).

A terceira possibilidade é baseada na associação de HHCY com doença vascular oclusiva, a qual pode ser mediada por danos na parede do vaso sanguíneo ou por coagulação sanguínea comprometida. Se esse fenômeno ocorrer no cérebro, os danos cérebro-vasculares induzidos pela Hcy podem levar a disfunção e degeneração neuronal secundária (Quinn et al., 1997; Troen, 2005; van den Berg et al., 1995).

Esses mecanismos não são exclusivos um do outro, nem querem postular que a Hcy atue como a causa das desordens clinicamente reconhecidas do SNC. Pelo contrário, eles enfatizam a possibilidade de que altas concentrações plasmáticas de Hcy podem aumentar a vulnerabilidade cerebral para outros insultos que resultem em disfunções cognitivas e patologias no SNC (Troen, 2005).

1.2 CITOESQUELETO

As células devem ter a capacidade de organizar-se no espaço, reorganizar seus componentes internos, interagir mecanicamente com o meio ambiente, mover-se de um lugar a outro e apresentar uma correta morfologia, modificando sua forma quando necessário. Todas essas funções estruturais e mecânicas apresentam-se altamente desenvolvidas em células eucarióticas e são dependentes de um extraordinário sistema de filamentos denominado citoesqueleto (Alberts, 2002).

Recentemente, a maioria dos estudos envolvendo o citoesqueleto enfoca sua relação com a transdução de sinais intracelulares. Tornou-se claro que o sistema celular de filamentos citoesqueléticos e as redes de vias de sinalização estão intimamente ligados e funcionam cooperativamente para gerar um fenótipo celular adaptado às condições imediatas da célula. Ademais, quando as células são remodeladas, provavelmente o citoesqueleto funciona como causa e efeito dessa remodelação: ele responde a sinais, ele organiza vias de sinalização no espaço celular e ele próprio pode exercer função de sinalização. Assim sendo, o mesmo poderia ser chamado de integrador da informação (Hollenbeck, 2001).

A notável rede do citoesqueleto é composta por três tipos de filamentos protéicos – filamentos de actina (microfilamentos) (MF), microtúbulos (MT) e filamentos intermediários (FIs) – cada um deles possuindo propriedades bioquímicas e biofísicas únicas (Figura 2) (Alberts, 2002; Carraway, 2000; Helfand et al., 2004).

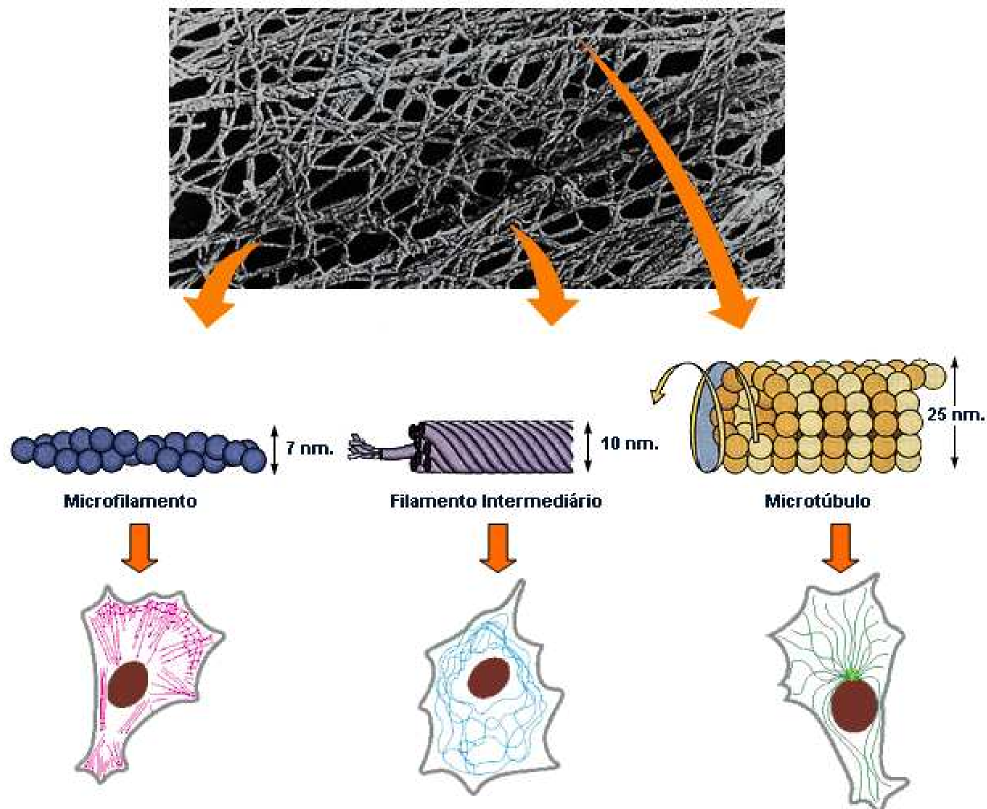

Figura 2. Representação esquemática dos constituintes do citoesqueleto. Adaptado de Alberts et al., 2002; www.uc.cl/sw/educ/biologia/bio100.

1.2.1 Filamentos de Actina

Os MFs são estruturas flexíveis com 5 a 9 nm de diâmetro distribuídos por toda a célula, porém, estão mais concentrados na região cortical, logo abaixo da membrana plasmática (Alberts, 2002).

A dinâmica da actina é essencial para mudanças na forma e motilidade celular em resposta a estímulos externos. Em neurônios em desenvolvimento, o citoesqueleto de actina tem um papel fundamental na formação, extensão e ramificação dos neuritos, bem como na sinaptogênese (Bloom et al., 2003; Fifkova e Delay, 1982; Hirokawa et al., 1989; Landis et al., 1988; Matus et al., 1982). Ademais, a regulação dos MF contribui significativamente na transmissão sináptica basal e nas várias formas de plasticidade sináptica, podendo assumir

um papel de integradora entre forma e função celular a nível da sinapse (Dillon e Goda, 2005).

Um importante processo celular, pelo qual a dinâmica de associação e desassociação dos MF é requerida, é a formação de protrusões de membrana (Pollard e Borisy, 2003) (Figura 3). A extensão de membrana melhor caracterizada é o lamelipódio, o qual é sustentado por uma rede ramificada e densa de F-actina com crescimento rápido em direção a membrana celular, garantindo a protrusão e a migração celular (Small, 1994). Quando os feixes de actina se estendem para além da borda do lamelipódio, formando estruturas semelhantes a agulhas, eles são chamados de "microspikes" ou filipódios (Small et al., 2002). Paralelamente a essa atividade protrusiva, o lamelipódio desempenha importantes papéis, como o envolvimento na formação de adesão celular ao substrato e em processos de pinocitose e fagocitose. Eles ainda recrutam todos os componentes requeridos nessas funções. Além disso, o processo de adesão celular por si só implica na reorganização dos lamelipódios, levando a formação de diferentes classes de complexos de adesão (Pantaloni et al., 2001).

Os filipódios, por sua vez, são responsáveis por detectar sinais no seu meio ambiente (Davenport et al., 1993; Goodhill et al., 2004), atuando como locais de transdução de sinal, interagindo com componentes da matrix extracelular para desencadear vias de sinalização que levarão a uma resposta celular adequada (Lidke et al., 2005). Nos cones de crescimento de axônios em migração, os filipódios buscam orientações no ambiente externo que permitirão aos axônios "navegarem" através de longas distâncias a procura do alvo adequado (Tessier-Lavigne e Goodman, 1996). Outro exemplo da importância biológica dos filipódios é a participação dos mesmos na formação das sinapses. Acredita-se que a sinaptogênese inicial pode resultar do contato entre filipódios e axônios (Dailey e Smith, 1996; Jontes e Smith, 2000; Lohmann et al., 2002; Ziv e Smith, 1996).

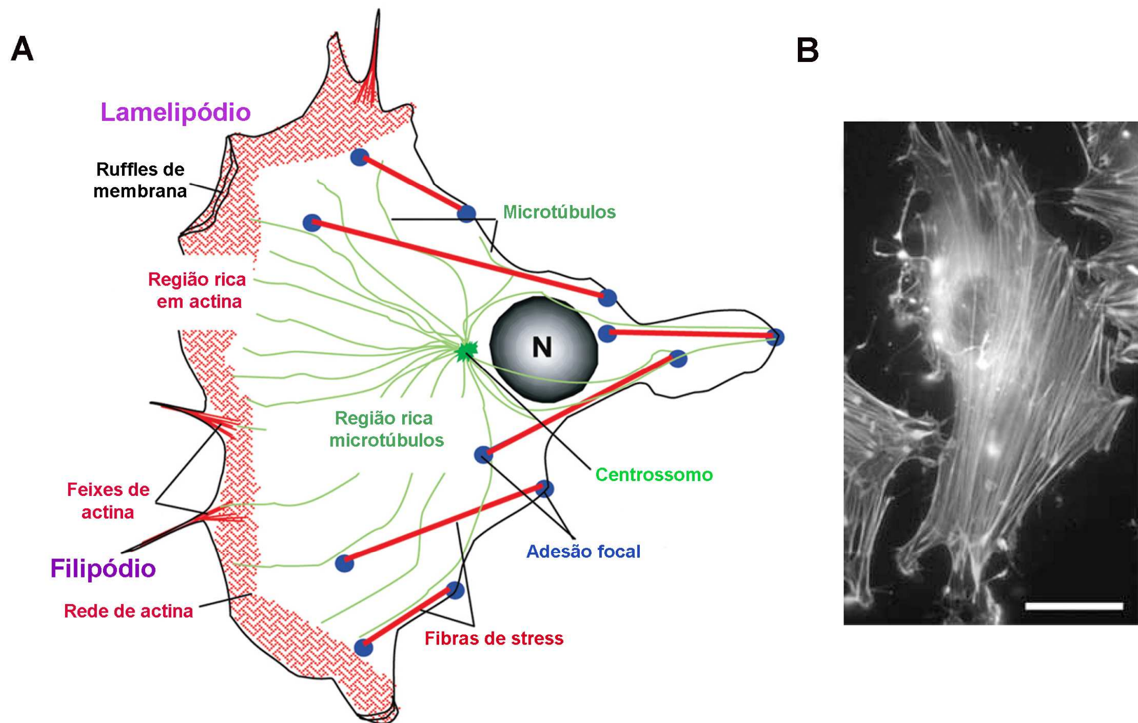

Figura 3: Representação esquemática da distribuição dos filamentos de actina. Adaptado de Etienne-Manneville, 2004 (Etienne-Manneville, 2004) e Wehrle-Haller e Imhof, 2003 (Wehrle-Haller e Imhof, 2003).

Diversas evidências relacionam alterações na dinâmica dos filamentos de actina com condições neurodegenerativas. Por exemplo, na doença de Alzheimer os filamentos de actina podem ter sua expressão e funções alteradas, contribuindo para o prejuízo de memória e perda sináptica decorrentes da doença (Butterfield et al., 2006; Sultana et al., 2006). Outro relevante exemplo são os corpos de Hirano, os quais são inclusões citoplasmáticas ricas em actina, descritos freqüentemente em uma variedade de condições neurodegenerativas (Davis et al., 2008; Kim et al., 2009; Maselli et al., 2003).

1.2.2 Microtúbulos

Os MTs são polímeros longos e rígidos, formados por subunidades protéicas de tubulina. Possuem diâmetro em torno de 25 nm e se estendem ao longo de todo o citoplasma.

A subunidade formadora dos MT é a tubulina, um heterodímero formado por duas proteínas globulares bastante similares denominadas α e β tubulina, fortemente ligadas por ligações não-covalentes. Os MTs possuem uma extremidade ligada ao único centro organizador de microtúbulos da célula, chamado centrossomo. A partir desta estrutura os microtúbulos estendem-se por toda a célula (Diaz et al., 1998; Downing, 2000).

Os MTs têm papel fundamental nos movimentos celulares, transporte intracelular de organelas e formação do fuso mitótico durante a divisão celular (Nogales, 2000; Sanchez et al., 2000; Schulze et al., 1987). No cérebro, estão envolvidos em várias funções celulares, incluindo transporte axonal, sinaptogênese (Vale et al., 1985), crescimento e elongação de neuritos (Riederer et al., 1997).

1.2.3 Filamentos Intermediários

Das três fibras citoesqueléticas, os FIs são os mais diversificados, sendo codificados por aproximadamente 70 genes em humanos (Human Intermediate Filament Mutation Database; http://www.interfil.org). Os FIs são classificados em cinco grandes famílias, conforme os tipos celulares e teciduais, bem como o nível de diferenciação e de desenvolvimento. Famílias I-IV são localizadas no citoplasma celular, enquanto o tipo V, as lâminas nucleares, são importantes organizadores do envelope nuclear e do carioplasma (Parry et al., 2007). A Figura 4 ilustra a ocorrência e classificação dos FIs em humanos.

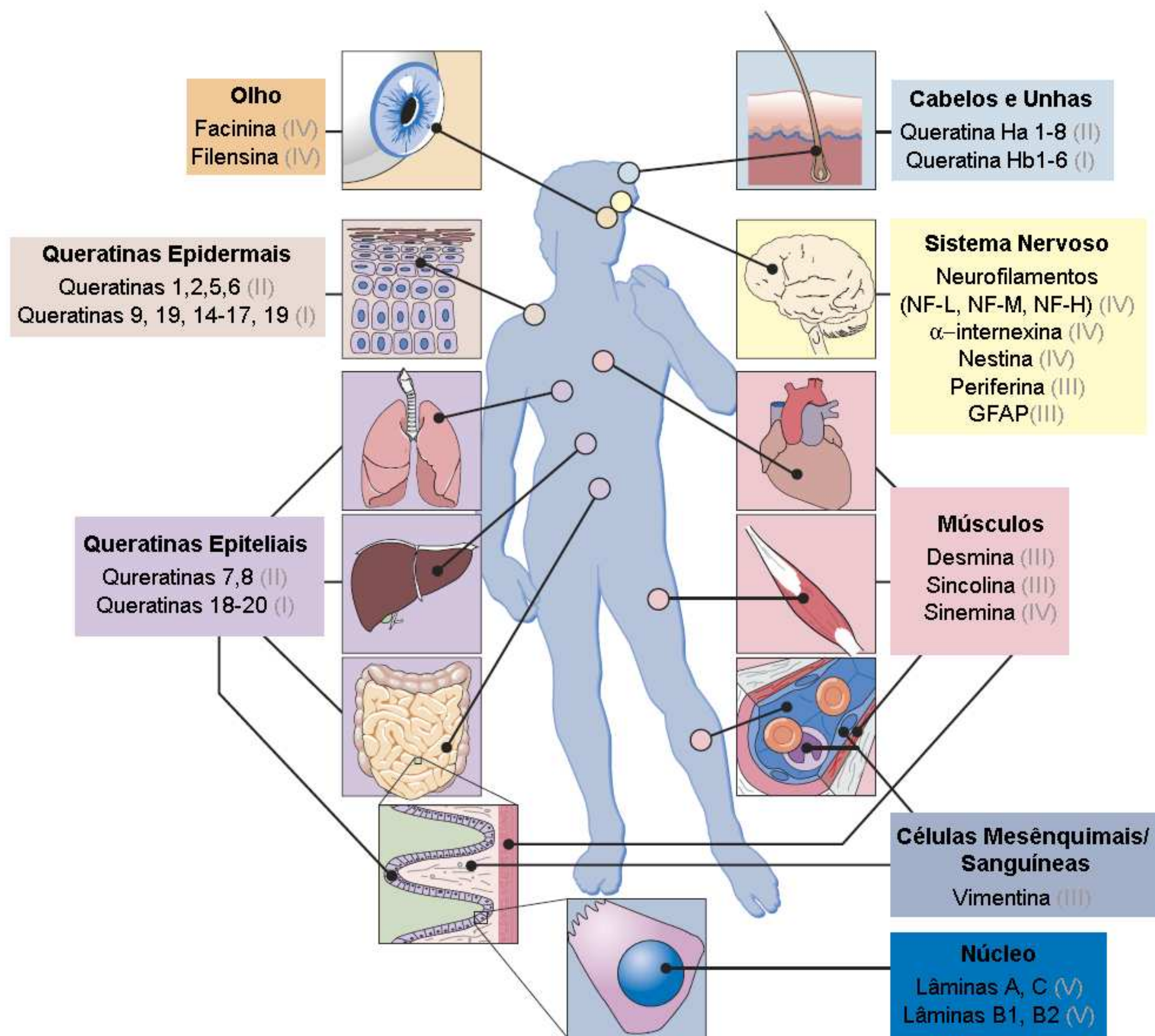

Figura 4. Classificação e ocorrência dos filamentos intermediários. Os algarismos romanos indicam a família dos FI a qual a proteína pertence. Adaptado de Toivola et al (2005).

Os FIs são fibras flexíveis com aproximadamente 10 nm de diâmetro, um tamanho intermediário entre MFs e MTs, sendo inicialmente considerados estruturas bastante estáticas e rígidas, com funções relacionadas a integridade estrutural de células e tecidos (Herrmann et al., 2007; Ivaska et al., 2007; Kim e Coulombe, 2007). De fato, o papel dos FIs em conferir resistência celular ao estresse mecânico e não mecânico é muito relevante e a conseqüente perda dessa função pode estar relacionada com doenças envolvendo fragilidade celular (Fuchs e Cleveland, 1998; Omary et al., 2004). No entanto, nos últimos anos as funções atribuídas aos FIs tem se ampliado drasticamente, pois os mesmos têm sido relacionados com

uma variedade de eventos celulares. Recentes evidências têm demonstrado o envolvimento de FIs na adesão e migração celular (Ivaska et al., 2007), na definição do posicionamento e forma de organelas celulares (Toivola et al., 2005) e na modificação de vários processos celulares, como a resposta ao estresse e crescimento de tecidos, devido a sua habilidade em regular moléculas de sinalização (Pallari e Eriksson, 2006). Outra função emergente dos FIs é a capacidade de funcionar como uma "âncora", que liga e regula a atividade de diversos efetores protéicos como receptores, proteínas quinases e proteínas de choque térmico (DePianto e Coulombe, 2004; Lee et al., 2001; Zastrow et al., 2004).

Como os FIs contribuem para o desenvolvimento celular e tecidual, doenças resultantes de modificações na estrutura e na dinâmica dos FIs são caracterizadas por diversos e graves fenótipos (Godsel et al., 2008; Magin et al., 2004; Pekny e Lane, 2007), que vão desde queratinopatias (Lane e McLean, 2004) até doenças relacionadas ao envelhecimento, distrofias musculares (Worman e Bonne, 2007) e doença de Parkinson (Kruger et al., 2003).

1.2.3.1 Neurofilamentos

Neurônios expressam várias proteínas de FIs dependendo do seu estágio de desenvolvimento e localização no sistema nervoso, são elas: nestina, três subunidades de neurofilamentos (NFs) - de baixo, médio e alto peso molecular - (NF-L, MF-M, e NF-H, respectivamente), α-internexina, periferina e sinemina (Ishikawa et al., 1968; Izmiryan et al., 2006; Julien e Mushynski, 1998; Kaplan et al., 1990; Portier et al., 1983).

A associação dos NFs ainda não é completamente entendida, no entanto, estudos *in vivo* têm demonstrado que os NFs são heteropolímeros, sendo a NF-L responsável pela formação do filamento propriamente dito (Ching e Liem, 1993; Lee et al., 1993). NF-M participa da formação de projeções laterais, estabilização da rede de filamentos e extensão

longitudinal (Elder et al., 1998; Elder et al., 1999a; Elder et al., 1999b; Jacomy et al., 1999). O NF-H também contribui com a formação de projeções laterais e pode interagir com outros elementos do citoesqueleto (Elder et al., 1998; Elder et al., 1999b; Jacomy et al., 1999). Recentes evidências sugerem que a α-internexina também pode ser incorporada ao NF maduro (Yuan et al., 2006). Periferina, um IF do tipo III, é expressa em neurônios motores, autônomos e sensoriais e também se associa com as subunidades dos NFs (Beaulieu et al., 1999a; Beaulieu et al., 1999b).

Como um constituinte do citoesqueleto celular, os NFs trabalham junto com MT e MF para aumentar a integridade estrutural, forma e motilidade celular, auxiliar na manutenção da morfologia neuronal e participar do transporte axonal de metabólitos do corpo celular até os terminais nervosos, processo este que é regulado por fosforilação (Ackerley et al., 2000; Beaulieu et al., 1999b; Jung et al., 2000; Kirkpatrick, 1999). A maior função dos NFs é o controle do calibre axonal, atividade que também é relacionada com a fosforilação. Essa função é extremamente importante, uma vez que a velocidade da condutividade de um impulso elétrico é proporcional ao seu calibre. NFs são particularmente abundantes em neurônios com grande diâmetro (>5 μM), como os neurônios periféricos motores, onde a rápida condução do impulso é crucial para o seu funcionamento adequado (Ohara et al., 1993; Sakaguchi et al., 1993).

É bem estabelecido que o acúmulo de NFs seja um marco para várias doenças neurodegenerativas, como a esclerose lateral amiotrófica, a doença de Alzheimer, a doença de Parkinson, a doença de Charcot-Marie-Tooth e a neuropatia axonal gigante (Bomont et al., 2000; Hirano et al., 1984; Schmidt et al., 1996; Schmidt et al., 1997; Shepherd et al., 2002; Watson et al., 1994). Os efeitos deletérios desses acúmulos podem ser visualizados quando as inclusões protéicas em axônios bloqueiam mecanicamente o transporte de partículas do

axônio, o qual eventualmente culminará em morte neuronal (Grant e Pant, 2000; Lariviere e Julien, 2004; Xu et al., 1993).

1.2.3.2 Proteína glial fibrilar ácida (GFAP)

Algumas células gliais, como os astrócitos maduros, sintetizam GFAP, no entanto o principal FI expresso em astrócitos imaturos, ou não diferenciados, é a vimentina (Langbein et al., 2005). Os astrócitos podem modificar a expressão de seus FIs em resposta a condições patológicas que afetam o cérebro, medula espinhal ou retina, como neurotrauma, isquemia e doenças neurodegenerativas. O termo gliose reativa é comumente utilizado para descrever a ativação astrocitária no estresse e patologias que afetam o SNC. O aumento na expressão de GFAP e vimentina e a re-expressão de nestina são os marcos da gliose reativa (Pekny e Pekna, 2004; Pekny, 2006). Esse aumento na expressão da GFAP na gliose reativa evidencia a importante contribuição dessa proteína na modulação da motilidade e forma celular, bem como na estabilidade estrutural e principalmente resistência do SNC as injúrias (Eng et al., 2000; Nawashiro et al., 1998; Pekny et al., 1999). Um dos mais dramáticos fenótipos de doenças provocadas por mutações nos FIs está relacionado a GFAP. Essas mutações causam a doença de Alexander, uma desordem fatal do SNC que é evidenciada por devastadores distúrbios no desenvolvimento normal do cérebro e crânio (Li et al., 2002), caracterizados por inclusões citoplasmáticas de GFAP e pequenas proteínas envolvidas com estresse nos astrócitos, denominadas fibras de Rosenthal (Hsiao et al., 2005; Quinlan et al., 2007). Mutações na GFAP podem comprometer a resposta astrocitária ao estresse, e este é um dos mecanismos patológicos da doença de Alexander (Der Perng et al., 2006).

O perfil da expressão gênica de camundongos que super expressam GFAP humana revelou a presença de resposta ao estresse que inclui genes envolvidos no metabolismo da glutationa, detoxificação de peróxido, homeostase de ferro, ação de citocinas, sistema

complemento e resposta de genes de fase aguda e anormalidades no tráfego de vesículas intracelulares, eventos esses que evidenciam a GFAP como um importante alvo em inúmeras vias de sinalização (Hagemann et al., 2005).

1.2.4 Fosforilação das Proteínas do Citoesqueleto

A fosforilação proteica é uma modificação pós-traducional importante para a transdução de sinais. Está presente em todos os processos celulares básicos, incluindo o metabolismo, crescimento, divisão, diferenciação, motilidade, tráfego de organelas, transporte de membrana, contração muscular, imunidade, aprendizado e memória (Grant e Pant, 2000; Helfand et al., 2004).

As proteínas quinases catalisam a transferência de um grupamento fosfato do ATP para aminoácidos específicos: em eucariotos, normalmente são resíduos de serina, treonina e tirosina. As proteínas fosfatases são fosforiltransferases que catalisam a reação inversa, ou seja, a hidrólise do grupo fosfato ligado ao substrato protéico (Bibb, 1999) (Figura 5).

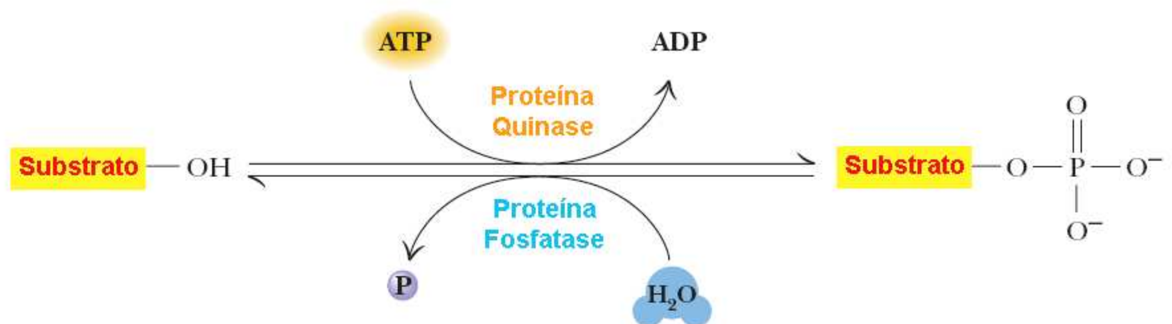

Figura 5. Representação esquemática de um sistema fosforilante. Adapatado de Garret e Grisham (2007).

Os FIs são componentes do citoesqueleto nos quais a fosforilação é o principal mecanismo responsável pela sua modulação, contribuindo na organização e função dos

mesmos de maneira célula e tecido-específicas (Grant e Pant, 2000; Kesavpanny, 2006; Omary et al., 2006), ao contrário dos MT e MF, cujos remodelamentos ocorrem primariamente via proteínas associadas (Burgess, 2005; Kawauchi et al., 2006) ou via outros mecanismos pós-traducionais (particularmente para MT) (Westermann e Weber, 2003).

A organização intracelular das redes de FIs é controlada por proteínas quinases e fosfatases (Chang e Goldman, 2004). Por exemplo, a fosforilação sítio-específica dos FIs pode induzir a desassociação dos FIs. O equilíbrio entre o estado fosforilado e não fosforilado dessas proteínas controla a contínua troca de subunidades entre as formas associados (filamentos) e não-associados (subunidades solúveis) dos FIs (Eriksson et al., 2004; Izawa e Inagaki, 2006).

Os FIs são substratos de inúmeras quinases, incluindo a proteína quinase C (PKC), a proteína quinase A (PKA), a quinase dependente de cálcio e calmodulina II (PKCamII), a proteína quinase ativada por mitógeno (MAPK) e a quinase Jun-amino-terminal (JNK). Entre outras. Estas quinases possuem um papel significativo na regulação da estrutura e associação dos FIs, bem nas interações entre FIs e suas proteínas associadas (Eriksson et al., 2004). Proteínas fosfatases como as pertencentes as subfamílias proteínas fosfatases 1, 2A e 2B (PP1, PP2A, PP2B) também desempenham uma importante contribuição na integridade dos citoesqueleto (Ceulemans e Bollen, 2004; Sontag, 2001).

Os NFs são as proteínas mais fosforiladas dos neurônios (Geisler et al., 1987; Goldstein et al., 1987; Jones e Williams, 1982; Julien e Mushynski, 1982; Lee et al., 1988; Pant e Veeranna, 1995). Esta fosforilação é topograficamente regulada, caracterizando-se por de uma intensa fosforilação em NF axonais e pouca ou nenhuma fosforilação em NF presentes no corpo celular e dentritos (Glicksman et al., 1987; Nixon et al., 1994; Nixon e Shea, 1992; Oblinger et al., 1987; Sternberger e Sternberger, 1983). Além disso, os sítios de fosforilação dos FIs podem ser alvo tanto de quinases dependentes de segundo mensageiros

(Sihag et al., 1988; Sihag e Nixon, 1989,1990) como de quinases independentes de segundo mensageiros (Julien e Mushynski, 1983; Sihag e Nixon, 1989,1990).

Distúrbios no sistema fosforilante do citoesqueleto de neurônios pode levar a morte neuronal e conseqüentemente ocasionar patologias graves. Um número elevado de doenças neurodegenerativas como doença de Alzheimer, demências, doença de Parkinson entre outras, são relacionadas com a fosforilação aberrante dos FIs, considerada um marco patológico dessas doenças (Goedert, 1998; Grant e Pant, 2000; Julien e Mushynski, 1982; Sihag et al., 2007). Outro importante exemplo é a esclerose amiotrófica lateral, uma desordem fatal marcada pela perda da atividade motora, a qual é caracterizada por agregados de NFs excessivamente fosforilados (Julien e Mushynski, 1998).

1.3 SINALIZAÇÃO CELULAR

Cada célula deve ser cuidadosamente regulada para satisfazer as necessidades do organismo como um todo (Cooper, 2001). A base para a coordenação das reações bioquímicas intracelulares é a comunicação intercelular, a qual possibilita a uma simples célula influenciar o comportamento de outras células de maneira específica (Krauss, 2003). Uma variedade de moléculas sinalizadoras são responsáveis pela regulação de todos os aspectos do comportamento celular, incluindo metabolismo, movimento, proliferação e diferenciação celular (Cooper, 2001).

As vias de sinalização celular envolvem mecanismos intra e extracelulares. As respostas aos diferentes tipos de sinalização celular podem ser rápidas ($<10^{-1}$ segundos), como fosforilação e outras modificações pós-traducionais, ou mudanças nas concentrações dos segundos mensageiros intracelulares, cálcio (Ca^{2+}) e AMP cíclico, bem como respostas mais lentas (de minutos a horas), tais como a regulação da transcrição gênica, migração

celular, controle do ciclo celular, proliferação celular e apoptose (Gilman et al., 2002; Sivakumaran et al., 2003; Weng et al., 1999).

O cérebro possui redes de sinalização celular em todos os níveis de sua organização, incluindo redes moleculares (no interior das células) e redes celulares (os circuitos neuronais), interconectadas para formar sistemas neuronais (por exemplo, o sistema límbico). Além disso, na medida em que as redes moleculares são conhecidas, tem-se demonstrado que proteínas podem funcionar como sinalizadoras através da alteração de sua conformação tridimensional e, conseqüentemente, exercer suas funções de acordo com as características físico-químicas do seu microambiente.

1.3.1 Cálcio

O Ca^{+2} é um sinal altamente versátil que pode regular diferentes funções celulares através da ação em várias vias de sinalização (Berridge et al., 2000; Carafoli et al., 2001). Na junção sináptica, por exemplo, o Ca^{+2} desencadeia exocitose dentro de microssegundos, enquanto no outro extremo, o Ca^{+2} funciona de minutos a horas para a condução de eventos como a transcrição gênica e a proliferação celular. Um grande desafio para a ciência é a compreensão de como essas inúmeras vias de sinalização desencadeadas pelo Ca^{+2} podem ser interconectadas para controlar os diversos processos celulares (Berridge et al., 2003).

O nível intracelular de Ca^{+2} é determinado por um equilíbrio entre sistemas que introduzem o Ca^{+2} no citoplasma (canais e transportadores) e reações inversas que removem esse íon pela ação combinada de tampões (bombas e trocadores iônicos). Durante a entrada de Ca^{+2} no citoplasma, uma pequena proporção de Ca^{+2} se liga a efetores que são responsáveis por estimular inúmeros processos Ca^{+2}-dependentes. Esses sistemas heterogêneos de sinalização são desencadeados por uma gama de mecanismos pelos quais o Ca^{+2} pode agir (Berridge et al., 2003).

O citoesqueleto de actina pode ser um alvo primário da sinalização desencadeada pelo Ca^{+2}, observando-se uma importante participação de quinases dependentes de Ca^{2+}, como PKCamII e MAPKs regulando a atividade de proteínas associadas a actina, modulando a organização da rede de MF durante a ramificação dos neuritos (Dent e Gertler, 2003; Kornack e Giger, 2005).

Doenças neurodegenerativas como doença de Alzheimer, doença de Parkinson, doença de Huntington, esclerose múltipla e esclerose amiotrófica lateral são caracterizadas por mecanismos patológicos que, em grande parte, convergem para distúrbios na homeostase do Ca^{+2} (Camins et al., 2006; Saez et al., 2006). Nosso laboratório possui inúmeros trabalhos relacionando alterações no sistema fosforilante associado aos FIs com a homeostase do Ca^{+2} (de Freitas et al., 1996; Funchal et al., 2005a; Funchal et al., 2005b; Zamoner et al., 2005; Zamoner et al., 2008a; Zamoner et al., 2008c; Zamoner et al., 2007). Os mecanismos que ocasionam essa perturbação na homeostase do Ca^{+2} são ilustrados na Figura 6. A caracterização molecular dessas vias de sinalização neurotóxicas dependentes de Ca^{+2} pode proporcionar alvos para futuras terapias farmacológicas ou genéticas de doenças neurodegenerativas (Arundine e Tymianski, 2003).

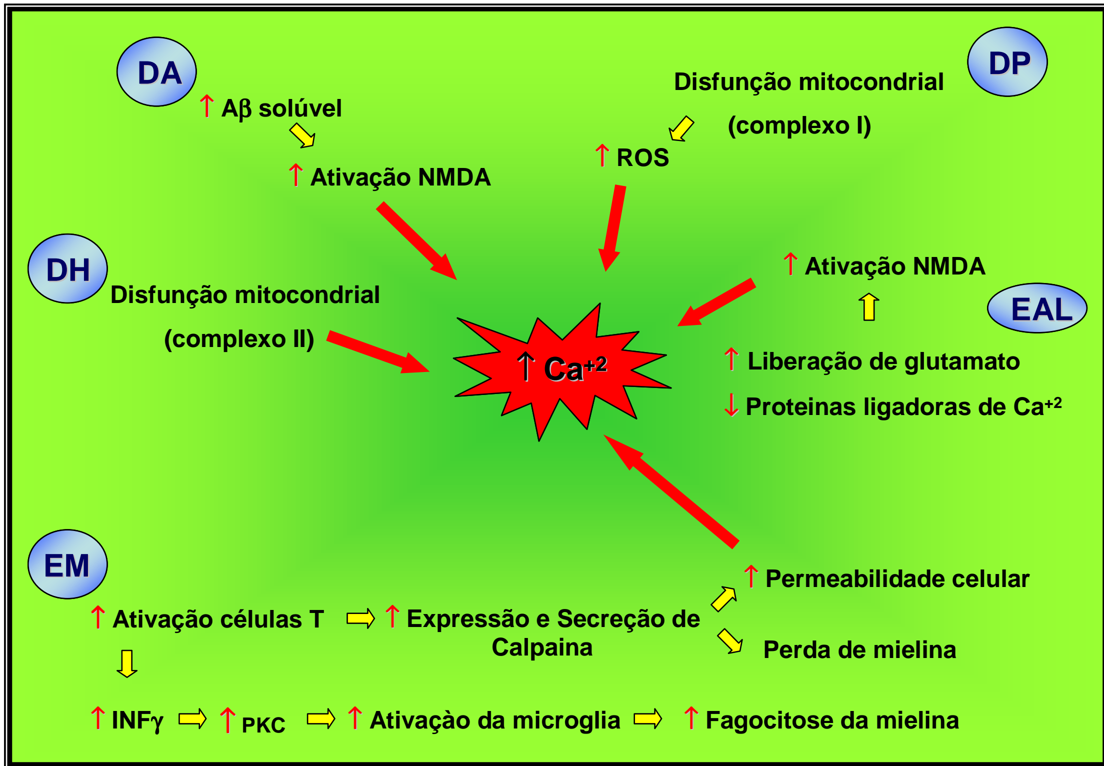

Figura 6: Mecanismos que aumentam o Ca^{+2} intracelular em algumas doenças neurodegenerativas. DA: doença de Alzheimer, DH: doença de Huntington, EM: esclerose múltipla, EAL: esclerose amiotrófica lateral, DP: doença de Parkinson, ROS: espécies reativas de oxigênio. Adaptado de Vosler et al. (2008).

1.3.2 Glutamato

A ação excitatória do glutamato no cérebro e medula espinhal de mamíferos foi demonstrada na década de 50 (Curtis e Watkins, 1960; Hayashi, 1952). Além de seu impacto imediato como um aminoácido excitatório, o glutamato desencadeia fenômenos plásticos vinculados à aprendizagem, memória (Attwell, 2000; Meldrum, 2000; Tapiero et al., 2002; Tzschentke, 2002) e plasticidade sináptica, incluindo a indução da potencialização de longa duração (LTP) e a depressão de longa duração (LTD), duas formas clássicas de plasticidade sináptica que são expressas em diversas áreas do cérebro (Artola e Singer, 1990; Gubellini et al., 2004; Ito, 1989). Além dessas funções, o glutamato está envolvido no desenvolvimento

de células nervosas, incluindo fenômenos de proliferação, diferenciação, migração e morte celular, assim como no envelhecimento (McDonald e Johnston, 1990; Meldrum, 2000; Segovia et al., 2001).

A dinâmica do citoesqueleto também pode ser alterada através de mecanismos glutamatérgicos. Os receptores glutamatérgicos podem mediar vias de sinalização dependentes de Rho-GTPases que controlam diretamente a reorganização da actina e a morfologia das espinhas dendríticas (Schubert et al., 2006). Além disso, nosso laboratório tem demonstrado alterações no sistema fosforilante dos FIs em diversas desordens neurodegenerativas envolvendo mecanismos glutamatérgicos (Funchal et al., 2002; Zamoner et al., 2008b).

A diversidade funcional do glutamato como neurotransmissor resulta da existência de uma grande variedade de receptores que coexistem em uma única sinapse. Os receptores glutamatérgicos podem ser classificados de acordo com suas propriedades farmacológicas e funcionais em receptores ionotrópicos (iGluRs) e metabotrópicos (mGluRs) (Monaghan et al., 1988; Nakanishi, 1994; Ozawa et al., 1998; Pin e Duvoisin, 1995).

1.3.2.1 Receptores Glutamatérgicos Ionotrópicos

Os iGluRs são canais iônicos que, quando ativos, tornam-se permeáveis a cátions como Na^+, Ca^{2+} e K^+. Podem ser classificados com bases farmacológicas em: receptores N-metil-D-aspartato (NMDA), (*R* ,*S*)-α-amino-3-hidroxi-5-metil-4-isoxazolepropionico ácido (AMPA) e cainato (KA), sendo os dois últimos freqüentemente chamados não NMDA (Meldrum, 2000; Ozawa et al., 1998; Tapiero et al., 2002).

Os receptores NMDA medeiam a transmissão sináptica lenta, são altamente permeáveis a Na^+, K^+ e Ca^{2+} (Lipton e Rosenberg, 1994; Ozawa et al., 1998), dependentes de voltagem e possuem glicina como co-agonista (Johnson e Ascher, 1992). Há evidências de

que a ativação excessiva destes receptores desfaz a homeostase do Ca^{2+}, levando a neurotoxicidade e ao dano neuronal causado pelo aumento do seu influxo. Existem mecanismos de lesão celular e subsequente morte por apoptose e/ou necrose que ocorrem como resultado do acúmulo intracelular de Ca^{+2} (Arundine e Tymianski, 2003).

Os receptores AMPA e KA medeiam a transmissão sináptica excitatória rápida e estão associados primariamente a canais independentes de voltagem, sendo permeáveis principalmente ao Na^{+} e K+, com baixa permeabilidade ao Ca^{2+} (Cotman et al., 1981). A ativação desses receptores não NMDA produz despolarização da membrana, ativando assim, indiretamente os canais de Ca^{+2} dependentes de voltagem (Cotman et al., 1981; Ozawa et al., 1998).

1.3.2.2 Receptores Glutamatérgicos Metabotrópicos

Os mGluRs pertencem a família dos receptores acoplados as proteínas ligantes de nucleotídeos da guanina (proteínas G) e, portanto, promovem a modulação de efetores intracelulares (segundos mensageiros), os quais ativam e/ou inibem diversos eventos de transdução do sinal celular (Ozawa et al., 1998; Pin e Duvoisin, 1995).

Os mGluRs são envolvidos com diversas funções no SNC de mamíferos. Essas funções incluem a regulação de canais de Ca^{+2} (Chavis et al., 1994; Sahara e Westbrook, 1993), a indução de LTP (Miura et al., 2002; Raymond et al., 2000) e LTD (Lin et al., 2000; Otani et al., 2002), a modificação da transmissão sináptica mediada por receptores NMDA (Awad et al., 2000; Guo et al., 2004; Harney et al., 2006; Pisani et al., 2001) e a regulação do desenvolvimento neuronal (Catania et al., 2001; Hannan et al., 2001; Pin e Duvoisin, 1995).

Além disso, diversas proteínas do citoesqueleto têm sido identificadas interagindo com mGluRs e dessa maneira modulando vias de sinalização desencadeadas pelos mesmos (Francesconi et al., 2009).

2. OBJETIVOS

2.1. OBJETIVO GERAL

Considerando que o citoesqueleto é um importante alvo para a sinalização celular em inúmeras doenças neurodegenerativas, o objetivo deste trabalho é investigar os possíveis efeitos tóxicos da Hcy sobre alguns parâmetros bioquímicos do citoesqueleto de células neurais, identificando algumas vias de sinalização envolvidas nestes efeitos.

2.2. OBJETIVOS ESPECÍFICOS

2.2.1 Estudar o efeito do tratamento crônico com Hcy sobre a expressão gênica, conteúdo total, fração citoesquelética e fosforilação dos FIs do citoesqueleto neural de córtex e hipocampo de ratos.

2.2.2 Realizar um estudo ontogenético dos efeitos do Hcy sobre a fosforilação *in vitro* dos FI em fatias de córtex cerebral e hipocampo de ratos.

2.2.3 Identificar vias de sinalização envolvidas nesses efeitos, com ênfase especial as proteínas quinases e fosfatases, bem como mecanismos envolvendo o glutamato e o cálcio intracelular;

2.2.4 Avaliar a ação da Hcy sobre a viabilidade celular e reorganização do citoesqueleto de actina e GFAP (em cultura primária de astrócitos corticais) e MTs (em cultura de neurônios corticais), e identificar a dependência de mecanismos envolvendo estresse oxidativo.

2.2.5 Verificar os efeitos da Hcy sobre a reorganização de MFs, MTs e FIs em cultura de células C6, bem como identificar mecanismos de ação envolvidos com tais efeitos.

2.2.6 Investigar os efeitos da Hcy sofre a fosforilação dos FIs em cultura de células C6 e identificar algumas vias de sinalização envolvidas.

3. MATERIAIS E MÉTODOS

A seção Materiais e Métodos está inserida nos quatro capítulos da na parte II desta tese.

PARTE II

Capítulo 1

HYPERHOMOCYSTEINEMIA SELECTIVELY ALTERS EXPRESSION AND STOICHIOMETRY OF INTERMEDIATE FILAMENT AND INDUCES GLUTAMATE- AND CALCIUM-MEDIATED MECHANISMS IN RAT BRAIN DURING DEVELOPMENT

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Running title: Homocysteine affects the cytoskeleton

Keywords: homocysteine; hyperhomocysteinemia; intermediate filaments; calcium; phosphatases; glutamate receptors

ABSTRACT

The aim of the present work was to investigate the actions of a chemically induced chronic hyperhomocysteinemia model on intermediate filaments (IFs) of cortical and hippocampal neural cells and explore signaling mechanisms underlying such effects. Results showed that in hyperhomocysteinemic rats the expression of neural IF subunits was affected. In cerebral cortex, glial fibrillary acidic protein (GFAP) expression was donwregulated while in hippocampus high and middle molecular weight neurofilament subunits (NF-H and NF-M, respectively) were upregulated. Otherwise, the immunocontent of IF proteins was unaltered in cerebral cortex while in hippocampus the immunocontent of cytoskeletal-associated low molecular weight neurofilament (NF-L) and NF-H subunits suggested a stoichiometric ratio consistent with a decreased amount of core filaments enriched in lateral projections. These effects were not accompanied by an alteration in IF phosphorylation. In vitro results showed that 500 μM Hcy induced protein phosphatases 1-, 2A- and 2B- mediated hypophosphorylation of NF subunits and GFAP in hippocampal slices of 17-day-old rats without affecting the cerebral cortex, showing a window of vulnerability of cytoskeleton in developing hippocampus. Ionotropic and metabotropic glutamate receptors were involved in this action, as well as Ca2+ release from intracellular stores through ryanodine receptors. We propose that the mechanisms observed in the hippocampus of 17-day old rats could support the neural damage observed in these animals.

INTRODUCTION

Tissue accumulation of homocysteine (Hcy) is the biochemical hallmark of homocystinuria, an inherited metabolic disorder caused by severe deficiency of cystathionine β-synthase (CBS, E.C.4.2.1.22) activity. Affected patients present alterations in various organs and systems, including central nervous and vascular systems (Kraus, 1998; Mudd, 2001). A variable degree of mental retardation and convulsions, whose pathophysiology is poorly understood, are also found in these patients (Malinow, 1990). There is a considerable body of evidence showing that an increase of Hcy levels in plasma is a strong and independent risk factor for the development of neurological and vascular diseases (Kuhn et al., 1998; Leblhuber et al., 2000; Mudd, 2001; Seshadri et al., 2002). In recent years, the mechanisms of Hcy-induced neural damage have been explored. It has been shown that Hcy inhibits Na+K+-ATPase activity (Matte et al., 2004; Streck et al., 2003b; Wyse et al., 2002), decreases energy metabolism (Streck et al., 2003a; Streck et al., 2003c) and induces oxidative stress (Kruman et al., 2000). Moreover, Hcy activates an apoptotic cell death pathway in cultured rat hippocampal neurons (Kruman et al., 2000) and its metabolite, homocysteic acid, can induce cell death in the rat hippocampus in vivo (Langmeier et al., 2003). Despite the efforts to understand the molecular basis of the neurological dysfunction accompagning homocystinuria, the effects of high Hcy levels on the cytoskeleton of neural cells is poorly known.

Intermediate filaments (IF) form extensive networks within the cytoplasm in most vertebrate cells. These networks extend radially in all directions coordinating cytoskeletal activities and relying information between the cell surface and the innermost compartments of the cell (Chang and Goldman, 2004). Neurofilaments (NF) are highly conserved neuronal IF expressed in adult neurons, characterized on the basis of molecular weight - a low

molecular weight 68-kDa isoform (NF-L), a middle molecular weight 160-kDa isoform (NF-M), and a highly phosphorylated high molecular weight 200-kDa isoform (NF-H). The assembly of the filamentous NF complex is dependent on the primary homopolymerization of NF-L, and the stoichiometry of NF subunits is highly regulated (Ching and Liem, 1993; Lee et al., 1993; Nixon and Shea, 1992). While the exact etiology of neurofilament (NF) aggregate formation remains to be determined, alterations in the stoichiometry of NF in a variety of motor neuron degenerations are associated with NF aggregate formation (Ge et al., 2003; Lariviere and Julien, 2004). Despite NFs have been universally considered to be composed of three subunits, experimental evidences demonstrate that the composition of NF could include other proteins. In this context, α-internexin has been described to coassemble with all three NF proteins into a single network in optic axons of adult mice (Yuan et al., 2006). Glial fibrillary acidic protein (GFAP) is an IF protein that is known to be localized in astrocytes, although its precise contributions to astroglial physiology and function are not clear. Typically, the role of IF scaffolding is to impart resistance to incident mechanical stress, and loss of function or mutations of numerous IF genes have been linked to several disease states involving cellular fragility (Coulombe et al., 2001; Fuchs and Cleveland, 1998; Herrmann and Aebi, 2004; Omary et al., 2006). IF proteins are known to be phosphorylated on their head and tail domains and the dynamics of phosphorylation/dephosphorylation play a major role in regulating the structural organization and function of IFs in a cell- and tissue-specific manner (Grant and Pant, 2000; Helfand et al., 2004; Jones and Williams, 1982; Julien and Mushynski, 1982; Ksiezak-Reding and Yen, 1987; Nixon and Lewis, 1986; Nixon and Sihag, 1991; Omary et al., 2006). There are increasing evidences that site-specific phosphorylation of IF proteins can affect their assembly, and structural organization (Sihag et al., 2007).

We have shown that 100 μM Hcy, a plasma concentration related with mild hyperhomocysteinemia, induced IF hyperphosphorylation in hippocampal slices of 17-day-old rat and this effect involved the activation of NMDA receptors, voltage-dependent calcium channels, Gi-coupled receptors and the stimulation of 45Ca2+ uptake (Loureiro et al., 2008). In agreement with this study, several mechanisms of toxicity have been reported including NMDA ionotropic receptor (iGluR) and group I metabotropic glutamate receptor (mGluR) mediated neurotoxicity (Ho et al., 2002; Zieminska et al., 2003). Animal models were used to better understand the pathophysiology of the diseases. In this context, Streck et al. (2002) have developed a chemical experimental rat model of hyperhomocysteinemia in which the plasma Hcy concentration is increased to levels that are similar to those found in human homocystinuria (Mudd, 2001). Thus, using this experimental model, we investigated the expression of NF and GFAP subunits as well as the immunoreactivity of these proteins in the high salt Triton X-100 insoluble and soluble fraction, representing respectively the ratio of polymerized/aggregated and soluble IF subunits in cerebral cortex and hippocampus of rats chronically injected with Hcy. Taking into account that the structural organization of the IF network is spatially and temporally regulated by phosphorylation (Janosch et al., 2000), we also investigated the effect of in vitro exposure of tissue slices to 500 μM Hcy, a concentration described to induce neural injury (Ferlazzo et al., 2008) on the IF-associated phosphorylating system and some molecular mechanisms that could support the alterations observed in the chemically induced hyperhomocysteinemia.

EXPERIMENTAL PROCEDURES

Radiochemical and compounds

[^{32}P]Na_2HPO_4 was purchased from CNEN, São Paulo, Brazil, D-L homocysteine, D-2-amino-5-phosphonopentanoic acid (DL-AP5), verapamil hydrochloride, dantrolene, calyculin A, okadaic acid, benzamidine, leupeptin, antipain, pepstatin, chymostatin, acrylamide, bis-acrylamide, antibodies anti NF-200 (clone N-52 and NE14), anti NF-150 (clone NN-18), anti NF-68 (clone NR-4), anti GFAP (clone G-A-5) and peroxidase conjugated rabbit anti-mouse IgG were obtained from Sigma (St. Louis, MO, USA). The potent competitive non-NMDA iGluR antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and the non-selective group I/group II mGluR antagonist (R,S)-a-methyl-4-carboxyphenylglycine (MCPG) were purchased from Tocris Neuramin (Bristol, UK). FK506 were obtained from Calbiochem (La Jolla, CA, USA). TRIzol Reagent, SuperScript-II RT pre-amplication system and Platinum Taq DNA polymerase were from Invitrogen. SYBRgreen was from Molecular Probe. The chemiluminescence ECL kit was obtained from Amersham (Oakville, Ontario, Canada). All other chemicals were of analytical grade.

Animals

Wistar rats (9, 12, 17, 21 and 29 days of age) from our breeding stock had free access to water and a 20% (w/w) protein commercial chow. They were maintained on a 12:12 h light/dark cycle and temperature of 22 ± 1°C. The “Principles of Laboratory Animal Care” (NHI publication no. 85–23, revised 1985) were followed in all the experiments and the experimental protocol was approved by the Ethics Committee for Animal Research of the Federal University of Rio Grande do Sul, Porto Alegre.

Homocysteine administration

Hcy was dissolved in saline buffer and the pH adjusted to 7.4. Hcy solution was administered subcutaneously twice a day at 8 h intervals from the 6th to the 28th day. Control animals received saline solution in the same volumes as those applied to Hcy-treated rats. Hcy doses were calculated from pharmacokinetic parameters previously determined (Streck et al., 2002). During the first week of treatment, animals received 0.3 μmol Hcy/g body weight. In the second week, 0.4 μmol Hcy/g body weight was administered to the animals, and in the last week rats received 0.6 μmol Hcy/g body weight. One hour after the last injection, the rats were killed by decapitation without anaesthesia. Treated animals achieved maximal plasma Hcy concentrations (0.40–0.50 mmol/L) 15 min after subcutaneous injection of Hcy, according to Streck et al. (2002).

Preincubation of tissue slices

Rats were killed by decapitation, the cerebral cortex and hippocampus were dissected onto Petri dishes placed on ice and cut into 400 μm thick slices with a McIlwain chopper. Tissue slices were initially preincubated at 30°C for 20 min in a Krebs-Hepes medium containing 124 mM NaCl, 4mM KCl, 1.2 mM $MgSO_4$, 25 mM Na-HEPES (pH 7.4), 12 mM glucose, 1 mM $CaCl_2$, and the following protease inhibitors: 1 mM benzamidine, 0.1 μM leupeptin, 0.7 μM antipain, 0.7 μM pepstatin and 0.7 μM chymostatin. In the experiments designed to study signaling mechanisms, slices were incubated in the presence or absence of 30 μM verapamil, dantrolene, 100 μM DL-AP5 and MCPG, 50 μM CNQX, 0.05, 0.5 and 5 μM okadaic acid, 200 nM calyculin A, 100 μM FK506 when indicated.

^{32}P-ortophoshate incorporation

After preincubation, the medium was changed and incubation was carried out at 30 oC with 100 µl of the basic medium containing 80 µCi of [^{32}P] orthophosphate with or without addition of verapamil, dantrolene, DL-AP5, MCPG, CNQX, okadaic acid, calyculin A or FK506 in the presence or absence of 100, 200, 300, 400 or 500 µM Hcy, when indicated. The labeling reaction was normally allowed to proceed for 30 min at 30 oC and stopped with 1 ml of cold stop buffer (150 mM NaF, 5 mM, EDTA, 5 mM EGTA, 50 mM Tris-HCl, pH 6.5, and the protease inhibitors described above). Slices were then washed twice with stop buffer to remove excess radioactivity.

Preparation of the high salt-Triton X-100 insoluble cytoskeletal fraction from tissue slices

After treatment, preparations of IF-enriched cytoskeletal fractions were obtained from cerebral cortex and hippocampus of rats as described by Funchal et al. (2003). Briefly, after the labelling reaction, slices were homogenized in 400 µl of ice-cold high salt buffer containing 5 mM KH_2PO_4, (pH 7.1), 600 mM KCl, 10 mM $MgCl_2$, 2 mM EGTA, 1 mM EDTA, 1 % Triton X-100 and the protease inhibitors described above. The homogenate was centrifuged at 15800 x g for 10 min at 4°C, in an Eppendorf centrifuge, the supernatant discarded and the pellet homogenized with the same volume of the high salt medium. The resuspended homogenate was centrifuged as described and the supernatant was discarded. The Triton-insoluble IF-enriched pellet, containing NF subunits and GFAP, was dissolved in 1% SDS and protein concentration was determined. The protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

Polyacrylamide gel electrophoresis (SDS-PAGE)

Equal protein concentrations of the cytoskeletal and soluble fractions were loaded onto 7.5 or 10 % polyacrylamide gels and analyzed by SDS-PAGE according to the discontinuous system of Laemmli (1970). After drying, gels were exposed to X-ray films (X-Omat XK1) at -70 oC with intensifying screens and finally the autoradiograph was obtained. Phosphorylation levels were quantified by scanning the films with a Hewlett-Packard Scanjet 6100C scanner and determining optical densities with an Optiquant version 02.00 software (Packard Instrument Company). Density values were obtained for the studies proteins.

Western blot analysis

Cytoskeletal and soluble fractions (50 μg) were analyzed by SDS-PAGE and transferred to nitrocellulose membranes (Trans-blot SD semi-dry transfer cell, BioRad) for 1 h at 15 V in transfer buffer (48 mM Trizma, 39 mM glycine, 20% methanol and 0.25% SDS). The nitrocellulose membranes were washed for 10 min in Tris-buffered saline (TBS; 0.5 M NaCl, 20 mM Trizma, pH 7.5), followed by 2 h incubation in a blocking solution (TBS plus 5% defatted dried milk). After incubation, the blot was washed twice for 5 min with TBS plus 0.05% Tween-20 (T-TBS), and then incubated overnight at 4°C in blocking solution containing the following monoclonal antibodies: anti NF-200 (clone N-52 and clone NE14), diluted 1:2000, anti NF-150 (clone NN-18) diluted 1:500, anti NF-68 (clone NR-4) diluted 1:1000 and anti GFAP (clone G-A-5) diluted 1:400. The blots were then washed twice for 5 min with T-TBS and incubated for 2 h in MTBS containing peroxidase conjugated rabbit anti-mouse IgG diluted 1:4000. The blots were washed twice again for 5 min with T-TBS and twice for 5 min with TBS. The blots were then developed using a chemiluminescence ECL kit. Autoradiograms and immunoblots were quantified by scanning the films with a Hewlett-

Packard Scanjet 6100C scanner and determining optical densities with an Optiquant version 02.00 software (Packard Instrument Company).

RNA extraction, cDNA synthesis and quantitative PCR

RNA was isolated from cerebral cortex and hippocampus slices using the TRIzol Reagent. Approximately 2 µg of total RNA were added to each cDNA synthesis reaction using the SuperScript-II RT pre-amplication system. Reactions were performed at 42ºC for 1 h using the primer T23V (5' TTT TTT TTT TTT TTT TTT TTT TTV). Quantitative PCR amplification was carried out using specific primer pairs designed with Oligo Calculator version 3.02 (http://www.basic.nwu.edu/biotools/oligocalc.html) and synthesized by IDT (MG, Brazil). The sequences of the primers used are listed in Table 1. Quantitative PCRs were carried out in an Applied-Biosystem 7500 real-time cycler and done in quadruplicate. Reaction settings were composed of an initial denaturation step of 5 min at 94ºC, followed by 40 cycles of 10 s at 94ºC, 15 s at 60ºC, 15 s at 72ºC and 35 s at 60ºC for data acquisition; samples were kept for 2 min at 40ºC for annealing and then heated from 55 to 99ºC with a ramp of 0.1ºC/sec to acquire data to produce the denaturing curve of the amplified products. Quantitative PCRs were made in a 20 µl final volume composed of 10 µl of each reverse transcription sample diluted 50 to 100 times, 2 µl of 10 times PCR buffer, 1.2 µl of 50 mM MgCl2, 0.4 µl of 5 mM dNTPs, 0.8 µl of 5 µM primer pairs, 3.55 µl of water, 2.0 µl of SYBRgreen (1:10,000 Molecular Probe), and 0.05 µl of Platinum Taq DNA polymerase (5 U/µl) (Invitrogen). All results were analyzed by the 2 -ΔΔCT method (Livak and Schmittgen, 2001). β-actin was used as the internal control gene for all relative expression calculations (Andrade et al, 2008). The Real-time PCR products were separated by 2.0% agarose gel electrophoresis and visualized with SyberGoldTM (Molecular Probes).

Statistical analysis

Data were analyzed statistically by Student's t-test or one-way analysis of variance (ANOVA) followed by the Least Square Difference (LSD) test when the F-test was significant. All analysis were performed using the SPSS software program on an IBM-PC compatible computer.

RESULTS

Initially we used Real-time PCR to analyze the effect of chronically injected Hcy on the expression of NF-L, NF-M, NF-H and GFAP, from cerebral cortex and hippocampus in animals sacrificed 1 h after the last Hcy injection. Results showed that in the cerebral cortex the expression of the three neuronal subunits was not altered while the expression of GFAP was down-regulated (Figure 1A). Conversely, in hippocampus, neither NF-L nor GFAP expression was altered, while the peripheral NF subunits, NF-M and NF-H, were up-regulated (Figure 1B). The immunocontent of the IF proteins recovered into the high-salt Triton X-100-insoluble cytoskeletal fraction and in the soluble fraction was also analyzed in the two cerebral structures. In cerebral cortex the immunocontent of NF-H, NF-M and NF-L was unaltered in both cytoskeletal and soluble fraction. Nonetheless, despite the down-regulated expression of GFAP, the immunocontent of this protein appeared to be unchanged in both the cellular fractions (Figure 2). On the other hand, in hippocampal slices, NF-L subunit, constituting the core of the filament, was down-regulated in the cytoskeletal fraction and up-regulated in the soluble fraction (Figure 3). Regarding the peripheral subunit NF-M, its immunocontent was increased in the soluble form, but unaltered in the cytoskeletal fraction (Figure 3). In addition, the immunocontent of NF-H as well as of GFAP was increased both in the cytoskeleton and in the soluble fraction (Figure 3).

When cortical and hippocampal slices from rats injected with Hcy were incubated with ^{32}P-orthophosphate, the phosphorylation level of NF-L, NF-M and GFAP was unaltered (Table 2). In addition, using a monoclonal antibody directed to a phosphorylated epitope of NF-H (clone NE14), we observed that the immunoreactivity was not altered either in the high-salt TritonX-100 insoluble and soluble fraction both in cerebral cortex and hippocampus slices (Figure 2 and 3).

We also investigated the in vitro effects of 500 μM Hcy on the phosphorylating system associated with the cytoskeletal fraction in cerebral cortex and hippocampus of rats during development. When cortical slices of 9, 12, 17, 21 and 29 day-old rats were incubated for 30 min with 500 μM Hcy, we did not observe any alteration of the phosphorylation level of NF-H, NF-M, NF-L and GFAP (Figure 4). On the other hand, exposure of hippocampal slices to Hcy significantly decreased the in vitro 32P incorporation into the IF proteins studied only in 17-day-old rats (Figure 4). A concentration-dependent curve using 100, 200, 300, 400 and 500 μM Hcy showed a biphasic effect, with hyperphosphorylation induced by 100 μM Hcy and hypophosphorylation induced by 500 μM Hcy (Figure 5)

Otherwise, Hcy-induced hypophosphorylation was prevented by calyculin A (200 nM), a PP1 and PP2A inhibitor or by different concentrations of OA, (0.05, 0.5 or 5 μM) described to inhibit PP2A, PP1 and PP2B, respectively (Figure 6). Interestingly, 0.5 μM OA per se, induced hyperphosphorylation of the cytoskeletal proteins, as compared with controls (Figure 6). In addition, FK 506, a specific PP2B inhibitor, also prevented the effect of Hcy on the cytoskeletal proteins (Figure 6).

Next, we examined the involvement of ionotropic and metabotropic glutamate receptors on the ability of Hcy to induce hypophosphorylation of the IF proteins. Results showed that the competitive NMDA ionotropic antagonist DL-AP5 (100 μM), the non-selective group I/group II metabotrpic glutamate receptor antagonist MCPG (100 μM), or the

competitive non-NMDA ionotropic glutamate receptor antagonist CNQX (50 μM), prevented the Hcy effect (Figure 7) .

In order to verify the role of intracellular Ca2+ in the Hcy-mediated decreased in vitro 32P incorporation into NF-H NF-M, NF-L and GFAP, hippocampal slices were co-incubated with 500 μM Hcy and dantrolene, an inhibitor of Ca2+ ryanodine receptors (RyR). Results showed that dantrolene, prevented the effect of Hcy (Figure 8). Moreover, to verify the involvement of Ca^{2+} influx through voltage-dependent calcium channels (VDCC) we used verapamil, the specific L-calcium channel (L-VDCC) inhibitor. Results showed that this compound was not able to prevent the effect of Hcy on the phosphorylating system, suggesting that Ca^{2+} influx via L-VDCC was not involved in the in vitro effect of 500 μM Hcy (Figure 8).

DISCUSSION

In the present study we first demonstrated that young rats submitted to a chronic hyperhomocysteinemia model presented an altered expression of IF subunits, reflecting induction of Hcy–mediated genomic mechanisms in the cerebral cortex and in the hippocampus of these animals. These findings provide an interesting insight on the differential susceptibility of cortical and hippocampal IF cytoskeleton to the chronic injury induced by this metabolite. These effects, are probably related to the specific physiological responses of cortical and hippocampal neurons and astrocytes to the insult. We could also suppose that Hcy interferred with the regulatory mechanisms that control gene expression of different IF subunits. The genes coding for NF proteins have been cloned in a number of species and are differentially regulated during development (Grant and Pant, 2000) and in neurodegenerative diseases (Thyagarajan et al., 2007). Consistent with NF expression in Hcy-

treated cerebral cortex, the immunocontent of cytoskeletal-associated and soluble subunits remained constant compared with the control. On the other hand, despite the down-regulated GFAP expression, the bulk of GFAP immunocontent in cerebral cortex was similar to controls. Moreover, in hippocampus, GFAP immunocontent was significantly increased, despite its unaltered expression in Hcy-treated animals. Conversely, in the hippocampus of chronically Hcy-injected rats the immunocontent of cytoskeletal-associated NF-L and NF-H subunits suggests a stoichiometric ratio consistent with a decreased amount of core filaments enriched in lateral projections. Otherwise, up-regulated immunocontent suggests an imbalance favoring soluble NF subunits.

The misregulation of the neural cytoskeleton could contribute to brain damage observed in this experimental model (Matte et al., 2007; Streck et al., 2003b; Streck et al., 2006). In this context, abnormalities in the stoichiometry of neuronal IF proteins may contribute to the formation of toxic IF inclusions typical of neurodegenerative diseases such as amyotrophic lateral sclerosis (Beaulieu et al., 1999; Julien and Beaulieu, 2000; Julien, 2001). Moreover, in cortical and hippocampal astrocytes, increased immunocontent/expression ratio of GFAP suggests a reduction in the turnover rate of this protein probably due to a failure of the ubiquitin-proteasome system (Mignot et al., 2007). In the case of hippocampus, we propose that this reduced turnover could lead to GFAP accumulation, found in neurodegenerative diseases characterized by astrocytes containing cytoplasmic aggregates, such as Alexander´s disease (Bachetti et al., 2008). Taken together, these results show that in rat hippocampus, cytoskeleton is more susceptible to hyperhomocysteinemia than in cerebral cortex.

Functional properties of the NF proteins such as cross-linking, NF assembly, axonal transport and protein-protein interactions are controlled locally in the axon by phosphorylation and dephosphorylation (Petzold, 2005). However, the altered stoichiometry

of NF subunits observed in the hippocampus at the end of the chronic treatment was not accompanied by altered IF phosphorylation. Therefore, to better understand the events underlying the effects observed in the in vivo treatment we developed an in vitro approach using hippocampal and cortical slices to dissect out biochemical steps of Hcy-induced signaling events in these regions of brain during development. We propose that the in vivo misregulation observed in the 29 day old animals could be a delayed consequence of the signaling pathways elicited by Hcy earlier during brain development.

We showed that 500 μM Hcy, a plasma concentration found in patients with severe homocystinuria and in rats subjected to chemically induced hyperhomocysteinemia (Mudd, 2001; Streck et al., 2002), significantly induced PP1-, PP2A- and PP2B- mediated hypophosphorylation of IF subunits in hippocampal slices of 17 day-old-rats. Otherwise, the concentration-dependent curve demonstrated that IF hypophosphorylation is a selective response to 500 μM Hcy, while hyperphosphorylation was shown to be induced by 100 μM Hcy, characteristic of mild homocystinuria, corroborating our previous results in 17-day-old rat hippocampal slices (Loureiro et al., 2008). This evidence highlight a period of postnatal development during which heightened cytoskeletal vulnerability of hippocampus to Hcy becomes apparent and emphasizes different responses according to the intensity of the insult. Although in the light of the current knowledge we are not able to understand the reasons leading to these dual actions, they could be ascribed to the differential activation of calmodulin, a Ca2+ binding protein responsible for mediating many of the actions of Ca2+ , including activation of Ca2+/calmodulin-dependent protein kinases or phosphatases (Selvakumar and Sharma, 2006). In this context, dual action of Hcy has been previously described for other biochemical parameters (Lee et al., 2002; Luchowska et al., 2005; Panganamala et al., 1986; Stazka et al., 2005; Zhang et al., 2005). The high susceptibility of hippocampus to the Hcy insult could be ascribed to the highest level of plasticity of this

structure in the brain. Hippocampus is particularly vulnerable to damage from a variety of insults (McEwen, 2001), as well as pathological situations (Siebzehnrubl and Blumcke, 2008), since it contains a population of neurons that are continuously generated from late embryogenesis through adulthood and new neurons seem to be more vulnerable than mature neurons (Danzer, 2008). On the other hand, it is difficult to establish the molecular basis responsible for the window of vulnerability to the insult of Hcy at 17 day old, however, we could suppose that these findings participate of the developmental plasticity implicaded in the maturation of the hippocampal network.

IF hypophosphorylation is in agreement with previous evidences showing that protein phosphatases are highly concentrated in the mammalian brain (Strack et al., 1997a; Strack et al., 1997b; Yoshimura et al., 1999) and pointing the cytoskeleton as a preferential target of the action of phosphatases both in physiological and pathological conditions. (Liu et al., 2008; Saito et al., 1995). Moreover, recent studies indicate that Hcy alters the levels of phospho-tau mostly through PP2A activity (Luo et al., 2007; Sontag et al., 2007; Yoon et al., 2007; Zhang et al., 2007; Zhang et al., 2008). In this context, increased levels of astrocyte PP2A and PP2B were identified in Alzheimer´s disease cerebral cortex and was considered as part of the astrogliosis seen in this disorder (Pei et al., 1997). Also, patients with Charcot-Marie-Tooth disease type 1 (CMT1) present NF hypophosphorylation. (Watson et al., 1994).

Previous evidence showed that Hcy toxicity in the CNS was related to increased cytosolic Ca^{2+} levels (Ho et al., 2003; Lipton et al., 1997). Accordingly, we have previously demonstrated that the effects of 100 µM Hcy on the hippocampal cytoskeleton was mediated by Ca2+ signaling mechanisms (Loureiro et al., 2008) and in the present report we show that PP2B, a Ca^{2+}-binding enzyme (Shibasaki et al., 2002), mediates the effects of 500 µM Hcy. Thus, we further investigated the roles of Ca^{2+} in the effect of 500 µM Hcy on the cytoskeleton. In this context, the rise in intracellular Ca^{2+} concentrations in response to a

stimulus could originate from a Ca^{2+} influx pathway, from release of Ca^{2+} from an internal store, or through a combination of these (Peuchen et al., 1996). Results showed that the action of 500 μM Hcy was related to the participation of Ca2+ influx through NMDA channels and mobilization of intracellular Ca^{2+} stores through RyR. Evidence in the literature points to a reciprocal regulation of ionic channel, receptor activities and cytoskeleton equilibrium through phosphorylation/dephosphorylation mechanisms. RyRs are regulated by PKA-mediated phosphorylation and PP1-/PP2A-mediated dephosphorylation (Marx et al., 2000). In addition, PP1 has been linked to the regulation of NMDA and AMPA receptors (Feng et al., 2000), otherwise, NMDA receptors physiologically modulate the phosphorylation level of NF-M (Fiumelli et al., 2008). Interactions of Hcy with NMDA glutamate receptors have been implicated in the actions of Hcy on the cytoskeleton (Loureiro et al., 2008; Robert et al., 2005). Therefore, we could propose that Hcy-activated protein phosphatases could modulate the receptor-mediated Ca^{2+} conductance. Also, evidence in the literature shows that activation of NMDA and mGluR participate of the excitotoxic action of Hcy in cerebellar granule neurons (Zieminska and Lazarewicz, 2006) and in rat lymphocytes (Boldyrev and Johnson, 2007). Accordingly, we are showing that the action of 500 μM Hcy is also dependent on the ionic currents of Na+ through AMPA receptors and mGluR activation. mGluRs are linked to diverse cytoplasmic signaling enzymes. Group I mGluRs stimulate phospholipase C activity and the release of Ca^{2+} from cytoplasmic stores (Bjornar Hassel, 2006) and this action could potentiate the Ca2+-mediated mechanisms supporting the effects of Hcy on the cytoskeleton. Taken together, our results showing that inhibition of iGluRs, mGluRs, or RyRs lead to the same effect on the cytoskeleton suggest the role of different mechanisms responsible for cytoplasmic Ca^{2+} fluctuations provoked by Hcy. Therefore, blocking extracellular Ca^{2+} influx or Ca^{2+} release from endoplasmic reticulum

stores, via RyRs or mGluR-mediated inositol 1,4,5-triphosphate formation, could prevent the actions of Hcy on the IF-associated phosphorylating system.

It is known that the frequency of the Ca^{2+} oscillations reflects the strength of the extracellular stimulus, and this frequency can be translated into a frequency-dependent cell response (Alberts, 2008). Thus the differential activation of cytoskeletal-associated protein kinases (Loureiro et al., 2008) or phosphatases could be related to the way cells sense the frequency of Ca^{2+} spikes elicited by the different Hcy concentrations and change their response accordingly. In addition, Ca^{2+} sensitive proteins change their activities regulating transcription of different sets of genes. In this kind of mechanism protein kinases act as a molecular memory device leading to long-lasting cell responses to a previously activated signaling pathway (Alberts, 2008). Therefore, we propose that the altered IF expression and misregulation of the neural cytoskeleton evidenced on the in vivo model could be a long-term consequence of the Ca^{2+}-mediated actions elicited by Hcy on the hippocampus of 17-day-old rats. Moreover, this effect could participate in the neural damage provoked by high Hcy levels with important implications in brain function. The present data provide a new insight in the pathogenesis of diseases linked to hyperhomocysteinemia.

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FIGURE LEGENDS

Figure 1. Quantitative Real-time PCR analysis in cortex (A) and hippocampus (B) slices of rats submitted to chronic homocysteine treatment. Animals were treated subcutaneously twice a day at 8 h intervals from the 6th to the 29th day with Hcy or saline solution (control). The expression of IF subunits was analyzed by real-time PCR, as described in Experimental Procedures. Data represent mean ± S.E.M of 6-8 animals in each group. Statistically significant differences from controls determined by Student's t-test are indicated: $*P < 0.01$. NF-H, high molecular weight neurofilament subunit; NF-M, middle molecular weight neurofilament subunit; NF-L, low molecular weight neurofilament subunit and GFAP, glial fibrillary acidic protein.

Figure 2. Effect of chronic homocysteine (Hcy) treatment on the immunoreactivity of cytoskeletal-associated and soluble IF proteins from cerebral cortex of rats. Animals were treated subcutaneously twice a day at 8 h intervals from the 6th to the 29th day with Hcy or saline solution (control). The immunoblotting was carried out with the monoclonal antibodies described in Experimental Procedures. Representative nitrocellulose membranes of four independent experiments are shown. NF-H, high molecular weight neurofilament subunit; P-NF-H, phosphorylated form of the high molecular weight neurofilament subunit; NF-M, middle molecular weight neurofilament subunit; NF-L, low molecular weight neurofilament subunit and GFAP, glial fibrillary acidic protein. The blots were developed using an ECL kit.

Figure 3. Effect of chronic homocysteine treatment on the immunoreactivity of cytoskeletal-associated and soluble IF proteins from rat hippocampus. Animals were treated subcutaneously twice a day at 8 h intervals from the 6th to the 29th day with Hcy or saline solution (control). The immunoblotting was carried out with the monoclonal antibodies described in Experimental Procedures. Results are reported as mean ± S.E.M. of 8-10 animals. Statistically significant differences from controls determined by Student's t-test are indicated: $^{*}P < 0.05$. NF-H, high molecular weight neurofilament subunit; P-NF-H, phosphorylated form of the high molecular weight neurofilament subunit; NF-M, middle molecular weight neurofilament subunit; NF-L, low molecular weight neurofilament subunit and GFAP, glial fibrillary acidic protein. The blots were developed using an ECL kit. Representative nitrocellulose membranes are shown.

Figure 4. Effect of Hcy on the in vitro phosphorylation of neurofilament subunits and glial fibrillary acidic protein of the cerebral cortex (A, C, E) and hippocampal (B, D, F) slices of 9, 12, 17, 21 and 29-day-old rats. Slices of cerebral cortex and hippocampus were incubated with 500 μM Hcy in the presence of 32P-orthophosphate. The cytoskeletal fraction was extracted and the radioactivity incorporated into the high molecular weight neurofilament subunit (NF-H), middle molecular weight neurofilament subunit (NF-M), low molecular weight neurofilament subunit (NF-L) and glial fibrillary acidic protein (GFAP) was measured as described in Experimental Procedures. Data are reported as means ± S.E.M. of 15–20 animals in each group and expressed as percent of controls. Statistically significant differences from controls, as determined by one-way ANOVA followed by LSD test, are indicated: $^{*}P<0.05$. Representative overexposed autoradiographs and immunoblots of cerebral cortex (C and E) and hippocampus (D and F) loaded in 10 % (C and D) or 7.5% (E and F) SDS-PAGE are shown.

Figure 5. Effect of different concentrations of Hcy on IF phosphorylation. Slices of hippocampus were incubated with 32P-orthophosphate in the absence or presence of Hcy at doses from 100, 200, 300, 400 and 500 μM Hcy, as described in Experimental Procedures. The cytoskeletal fraction was extracted, analyzed by SDS-PAGE and the radioactivity incorporated into NF-H, NF-M, NF-L and GFAP was measured by determining the optical density values for the band correspondending to each protein. Data are reported as means ± S.E.M. of 12 animals in each group and expressed as % of control. Statistically significant differences from controls, as determined by one-way ANOVA followed by LSD test, are indicated: *P<0.05.

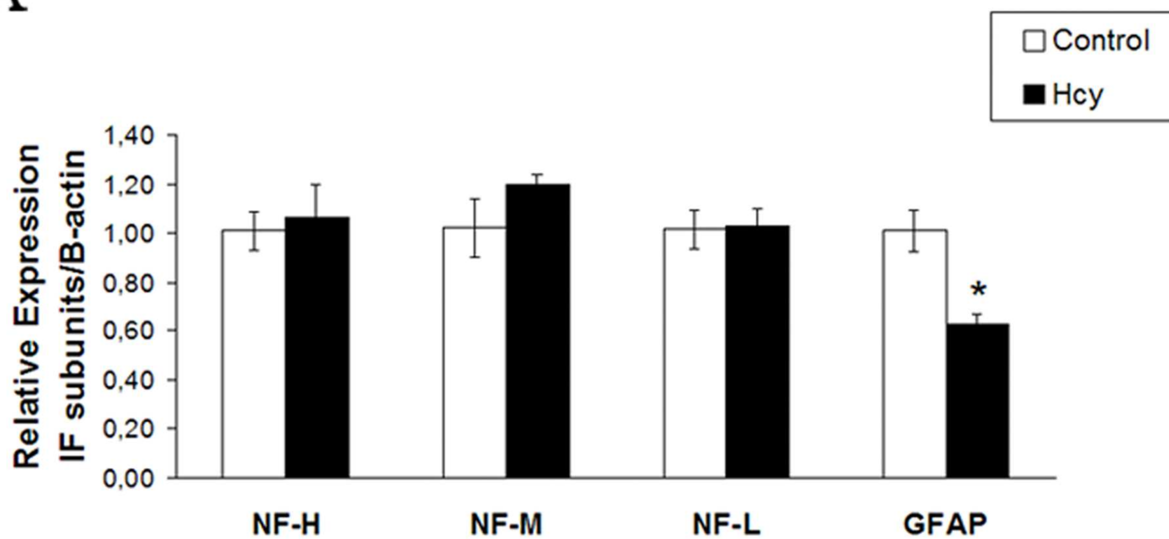
Figure 6. Involvement of protein phosphatases on Hcy-induced IF in vitro hypophosphorylation in hippocampus of 17 day old rats. (A) Participation of PP1, PP2A and PP2B was tested by using the protein phosphatase inhibitor okadaic acid (OA) at different concentrations: 0.05 μM, 0.5 μM, or 5 μM. (B) Participation of PP2A and PP1 on Hcy-induced IF hypophosphorylation was tested by using 200 mM calyculin A (Cal) and 100 μM FK506 was used for PP2B. Hippocampal slices were preincubated in the presence or absence of each inhibitor for 20 min and incubated with 32P-orthophosphate with or without 500 μM Hcy and/or inhibitors, as described in Experimental Procedures. The cytoskeletal fraction was extracted and the radioactivity incorporated into NF-H, NF-M, NF-L and GFAP was measured. Data are reported as means ± S.E.M. of 15 - 20 animals in each group and expressed as % of control. Statistically significant differences from controls, as determined by one-way ANOVA followed by LSD test are indicated: *P<0.001 compared with control group; #P<0.001 compared with Hcy group.

Figure 7. Effect of glutamate receptors on the action of Hyc in hippocampus of 17 day old rats. Hippocampal slices were preincubated for 20 min in the presence or absence of 100 μM DL-AP5, 50 μM CNQX, 100 μM MCPG and incubated with 32P-orthophosphate with or without the antagonists and/or 500 μM Hcy. The cytoskeletal fraction was extracted and the radioactivity incorporated into NF-H, NF-M, NF-L and GFAP was measured. Data are reported as means ± S.E.M. of 15 - 20 animals in each group and expressed as % of control. Statistically significant differences as determined by one-way ANOVA followed by LSD test are indicated: *P<0.05 compared with control group; #P<0.05 compared with Hcy group.

Figure 8. Effect of calcium-mediated mechanisms on the action of Hyc in hippocampus of 17 day old rats. Hippocampal slices were preincubated for 20 min in the presence or absence of 30 μM verapamil (Vera), 30 μM dantrolene (Dan) and incubated with 32P-orthophosphate with or without the blockers and/or 500 μM Hcy. The cytoskeletal fraction was extracted and the radioactivity incorporated into NF-H, NF-M, NF-L and GFAP was measured. Data are reported as means ± S.E.M. of 15-20 animals in each group and expressed as % of control. Statistically significant differences as determined by one-way ANOVA followed by LSD test are indicated: *P<0.01 compared with control group; #P<0.01 compared with Hcy group.

FIGURE 1

A



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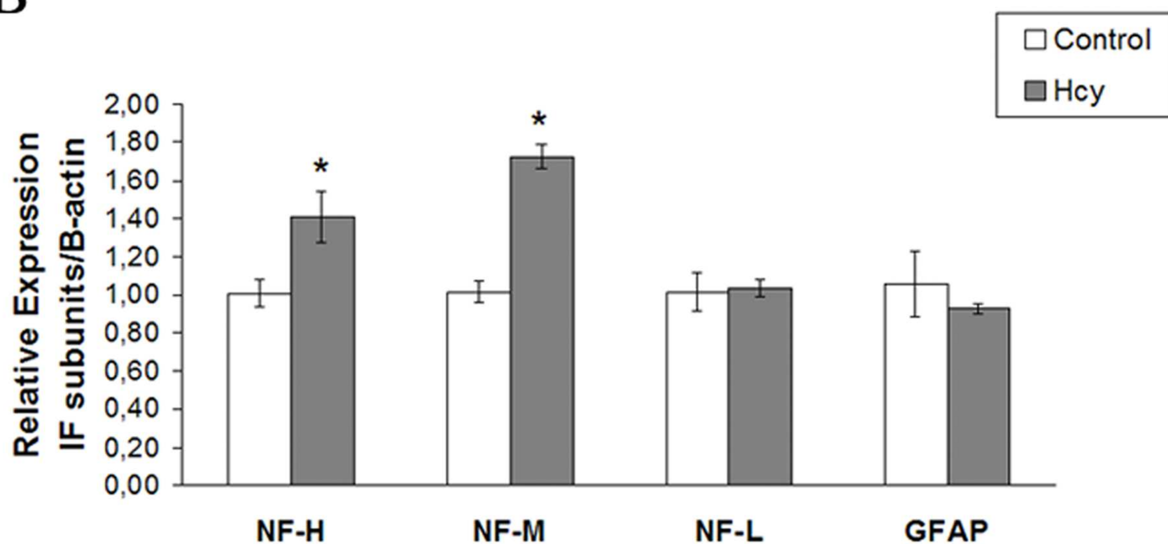

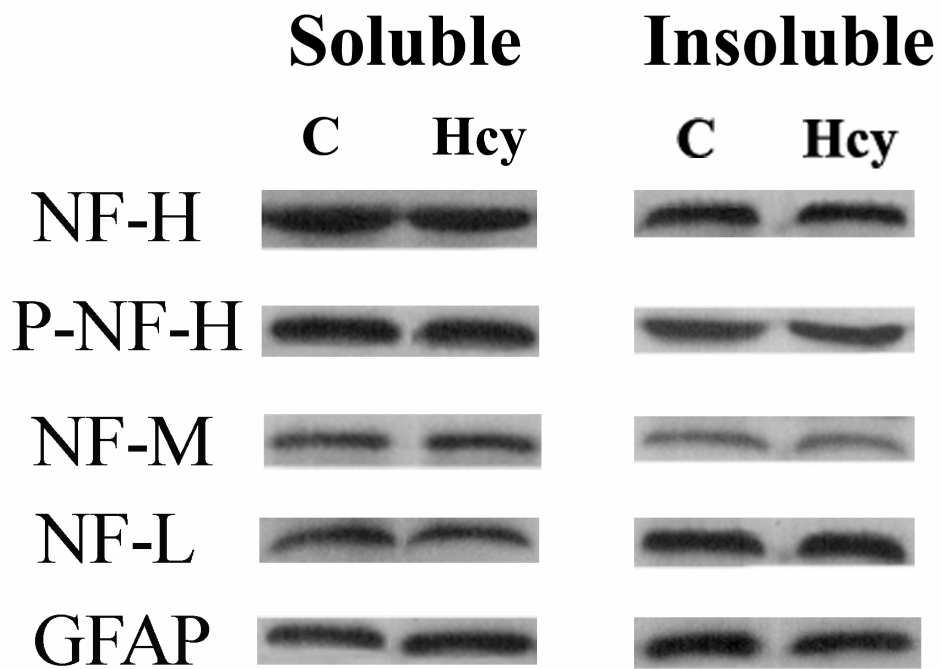
FIGURE 2
Soluble
Insoluble
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Hcy
C
Hcy
NF-H
P-NF-H
NF-M
NF-L
GFAP

FIGURE 3

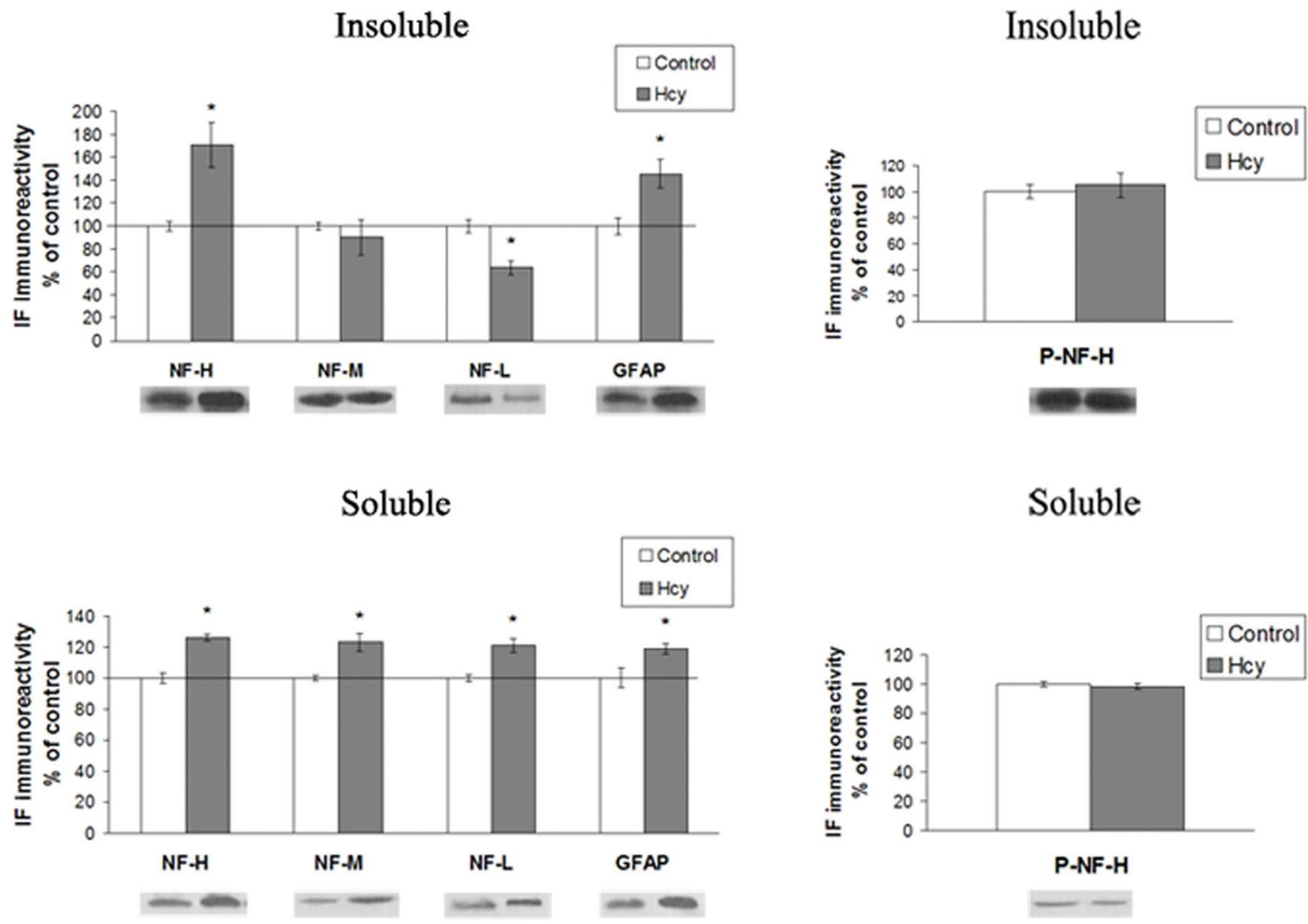
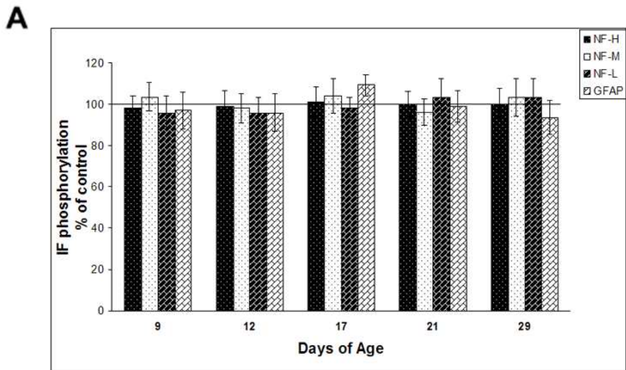
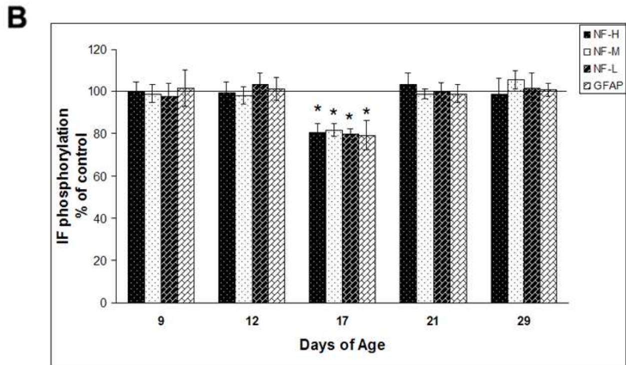
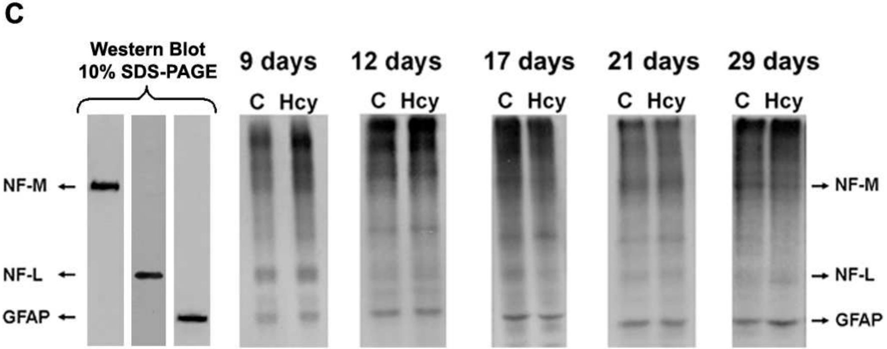
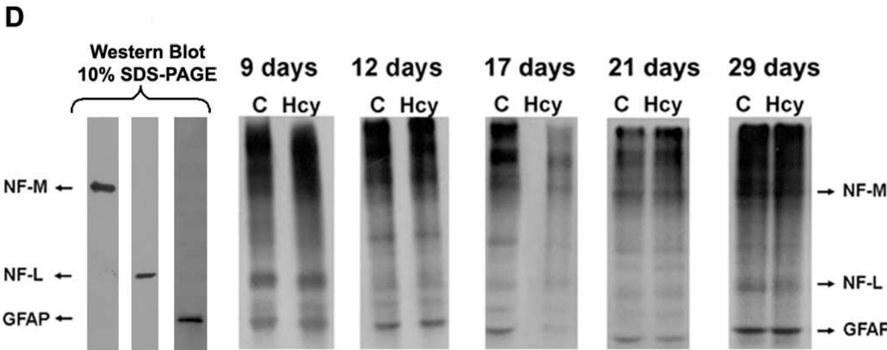


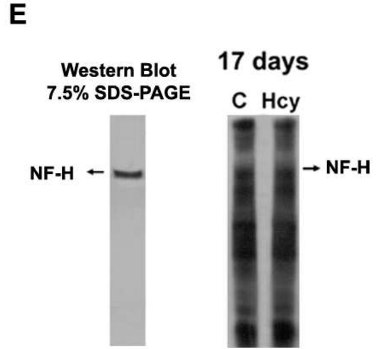
FIGURE 4











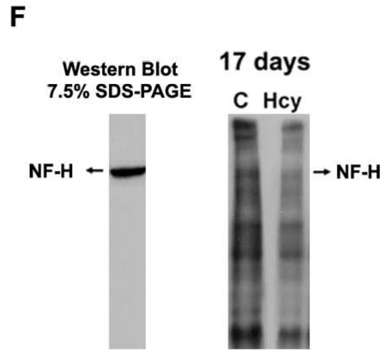


FIGURE 5

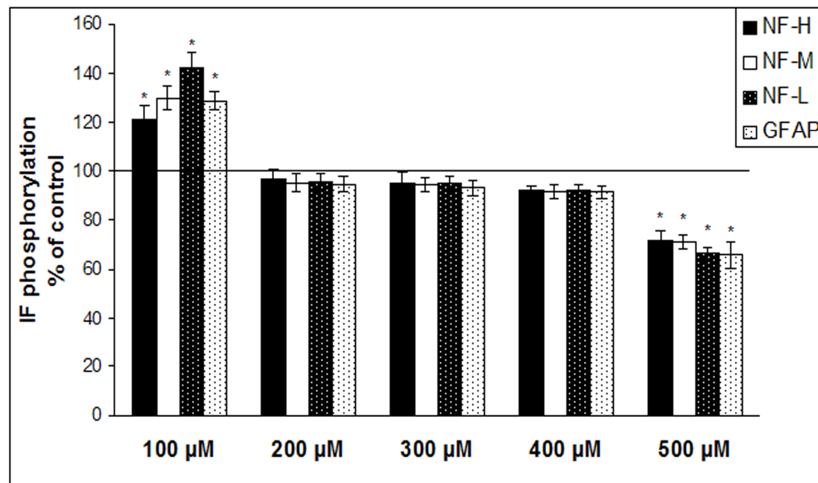
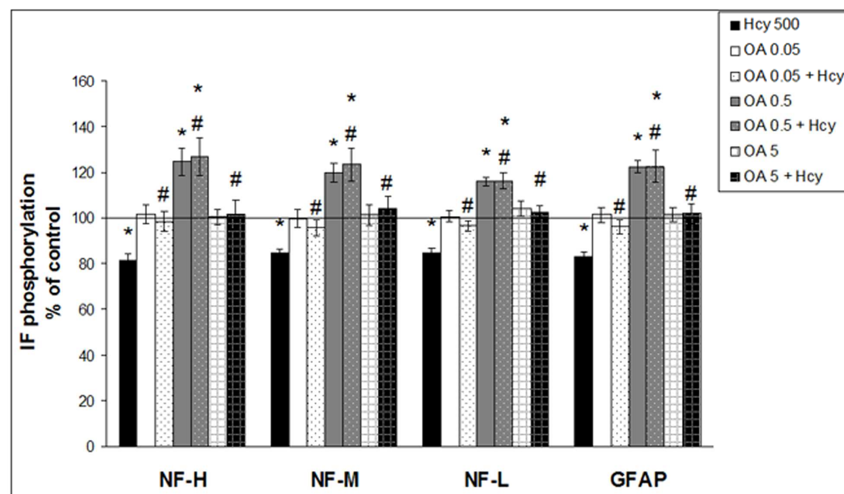


FIGURE 6

A



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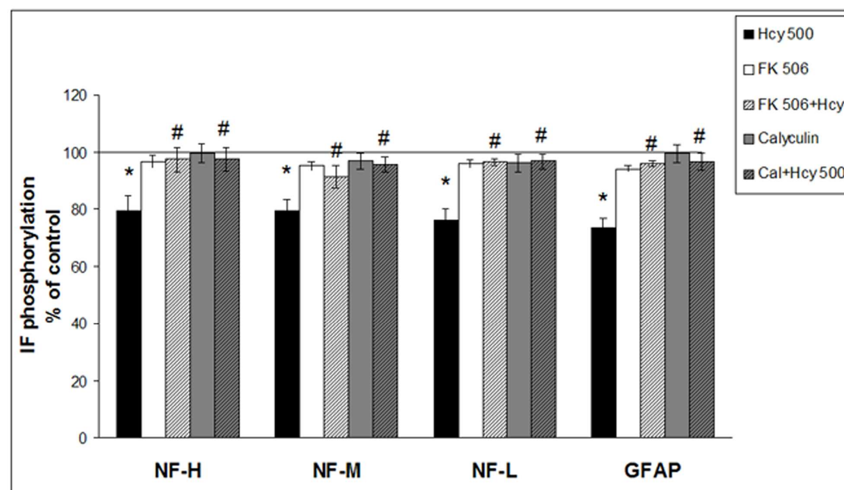

FIGURE 7

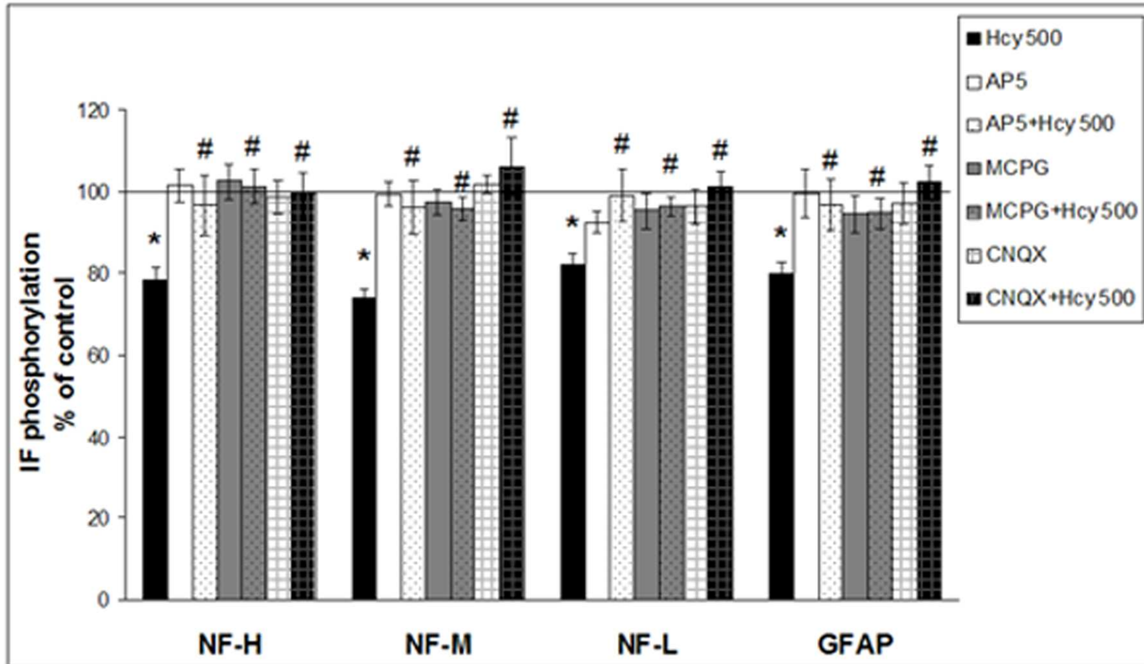

FIGURE 8

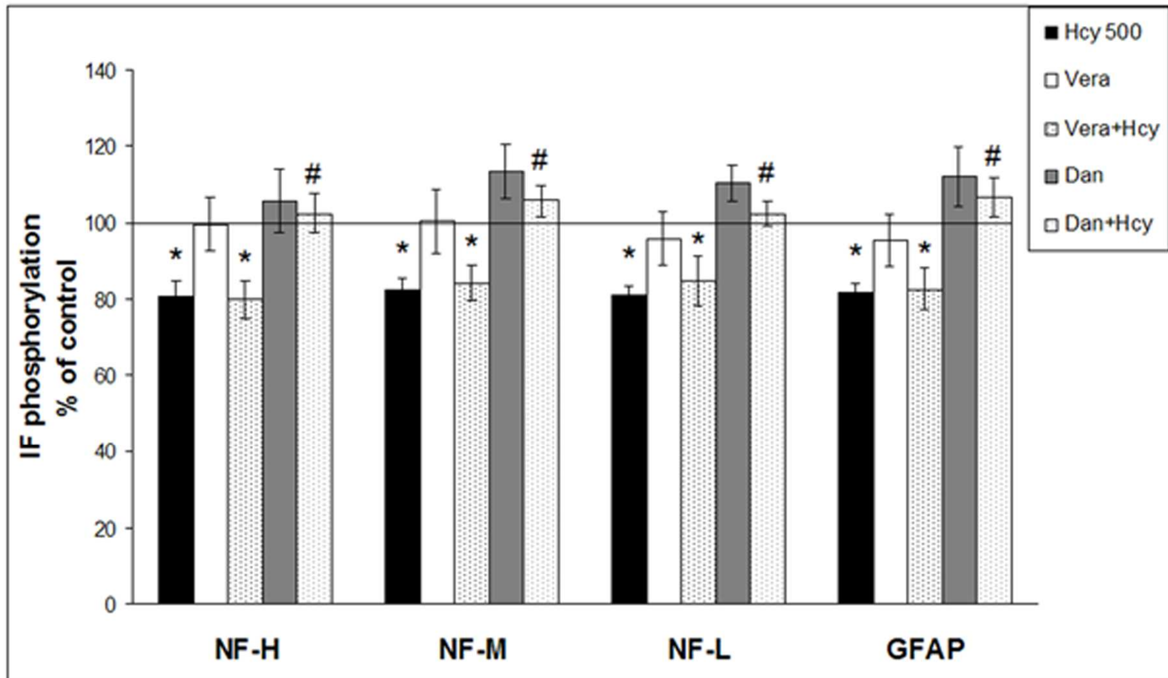

Table 1. Primers used for qPCR (quantitative polymerase chain reaction)

	Forward primer	Reverse primer
NF-H	5'ACCTATACCCGAATGCCTTCTT	5'AGAAGCACTTGGTTTTATTGCAC
NF-L	5'CCATGCAGGACACAATCAAC	5'CTGCAAGCCACTGTAAGCAG
NF-M	5'GAGATGTATTACGCAAAGTAC	5'CCAGTATGACCTTTATTGAGC
GFAP	5'CAGAAGCTCCAAGATGAAACCAA	5'TCTCCTCCTCCAGCGACTCAAC
β-Actin	5'TATGCCAACACAGTGCTGTCGG	5'TACTCCTGCTTCCTGATCCACAT

Table 2. Effect of chonically injected Hcy on the *in vitro* phosphorylation of intermediate filament subunits in brain of 29-day old rats

	IF Phosphorylation (% of control)							
	Cerebral Cortex				Hippocampus			
	Insoluble		Soluble		Insoluble		Soluble	
	Control	Hcy	Control	Hcy	Control	Hcy	Control	Hcy
NF-H	100.00 ± 3.64	101.97 ± 2.84	100.00 ± 3.82	99.02 ± 2.35	100.00 ± 8.71	105.68 ± 6.13	100.00 ± 3.91	108.93 ± 9.30
NF-M	100.00 ± 5.21	105.06 ± 4.78	100.00 ± 3.86	96.33 ± 1.77	100.00 ± 3.88	107.22 ± 6.33	100.00 ± 4.11	111.05 ± 10.29
NF-L	100.00 ± 2.75	108.24 ± 1.20	100.00 ± 4.14	95.44 ± 2.37	100.00 ± 3.39	113.57 ± 6.77	100.00 ± 3.05	112.43 ± 10.58
GFAP	100.00 ± 3.33	107.20 ± 3.14	100.00 ± 2.87	94.48 ± 3.65	100.00 ± 4.63	106.35 ±4.32	100.00 ± 3.02	114.13 ± 11.25

Results are reported as mean ± of 12-15 animals. Statistical analysis: Student-t test. NF-H, high molecular weight neurofilament subunit; NF-M, middle molecular weight neurofilament subunit; NF-L, low molecular weight neurofilament subunit and GFAP, glial fibrillary acidic protein.

Capítulo 2

HOMOCYSTEINE ACTIVATES CALCIUM-MEDIATED CELL SIGNALING MECHANISMS TARGETING THE CYTOSKELETON IN RAT HIPPOCAMPUS

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Homocysteine activates calcium-mediated cell signaling mechanisms targeting the cytoskeleton in rat hippocampus

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Abstract

Homocysteine is considered to be neurotoxic and a risk factor for neurodegenerative diseases. Despite the increasing evidences of excitotoxic mechanisms of homocysteine (Hcy), little is known about the action of Hcy on the cytoskeleton. In this context, the aim of the present work was to investigate the signaling pathways involved in the mechanism of action of Hcy on cytoskeletal phosphorylation in cerebral cortex and hippocampus of rats during development. Results showed that 100 μM Hcy increased the intermediate filament (IF) phosphorylation only in 17-day-old rat hippocampal slices without affecting the cerebral cortex from 9- to 29-day-old animals. Stimulation of $^{45}Ca^{2+}$ uptake supported the involvement of NMDA receptors and voltage-dependent channels in extracellular Ca^{2+} flux, as well as Ca^{2+} release from intracellular stores through inositol-3-phosphate and ryanodine receptors. Moreover, the mechanisms underlying the Hcy effect on hippocampus cytoskeleton involved the participation of phospholipase C, protein kinase C, mitogen-activated protein kinase, phosphoinositol-3 kinase and calcium/calmodulin-dependent protein kinase II. The Hcy-induced IF hyperphosphorylation was also related to G_i protein and inhibition of cAMP levels. These findings demonstrate that Hcy at a concentration described to induce neurototoxicity activates the IF-associated phosphorylating system during development in hippocampal slices of rats through different cell signaling mechanisms. These results probably suggest that hippocampal rather than cortical cytoskeleton is succeptible to neurotoxical concentrations of Hcy during development and this could be involved in the neural damage characteristic of mild homocystinuric patients.
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Keywords: Homocysteine; Intermediate filaments; Phosphorylation; Cell signaling; Hippocampus

1. Introduction

Homocysteine (Hcy) is a sulfur-containing amino acid formed during the intracellular conversion of methionine to cysteine. This toxic intermediate may be remethylated to form methionine or trans-sulfurated to give cysteine. In the trans-sulfuration pathway, Hcy and serine form cystathionine in a condensation reaction catalyzed by cystathionine beta synthase (CBS). Deficiency in CBS, methylenetetrahydrofolate reductase, methionine synthase or in vitamins B6, B12, and folate leads to abnormal homocysteine concentrations (Cattaneo, 1999). Normally Hcy concentration in human blood does not exceed 14 μM (Seshadri et al., 2002). Homocystinuria, an inborn errors of metabolism, or disturbances caused by dietary deficiency in folic acid and/or Vitamins B6 and B12, may lead to hyperhomocysteinemia in the range of 50–500 μM (Scott and Weir, 1998; Yudkoff, 1999; Lipton et al., 1997; Mudd et al., 2001).

In addition to the potentially fatal major cardiovascular clinical features, patients with homocystinuria display neurological abnormalities such as mental retardation, cerebral atrophy, seizures and a predisposition to schizophrenia and epilepsy (Grieco, 1977; Mudd et al., 1985; Van den Berg et al., 1995; Bleich et al., 2000, 2003; Levine et al., 2002; Sachdev et al., 2002). Hyperhomocysteinemia has also been associated with neurodegenerative diseases such as Alzheimer's and Parkinson's disease (Miller, 1999; Allain et al., 1995; Seshadri et al., 2002).

In recent years, the mechanisms of Hcy-induced damage have been explored in different cell types and tissues (Kruman et al., 2000; Streck et al., 2002, 2003, 2004; Langmeier et al.,

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2003; Cattaneo, 2003, 2006; Matté et al., 2004; Hron et al., 2007). Also, *in vitro* studies indicated that chronic Hcy neurotoxicity in primary cerebellar granule cell cultures was due to excitotoxic mechanisms mediated by NMDA receptors (Zieminska and Lazarewicz, 2006). In this context, Schwarz and Zhou (1991) postulated a pathologically elevated glutamatergic neurotransmission that may be caused by a Hcy-induced effect on glutamate receptors inducing Ca^{2+} influx through NMDA receptor activation leading to excitotoxicity (Lipton et al., 1997; Ho et al., 2002).

It is largely described that the rise in intracellular Ca^{2+} concentrations in response to a stimulus exerts an important influence in neuronal morphology by affecting the cytoskeleton. In this context, Rodnight et al. (1997) have described a role of Ca^{2+} on the phosphorylation state of the intermediate filament (IF) protein of the astrocytic cytoskeleton, glial fibrillary acidic protein (GFAP), in immature hippocampal slices. Moreover, we have previously described the involvement of Ca^{2+}-dependent mechanisms in the phosphorylation of neuronal and glial IF proteins from cerebral cortex of rats in experimental models of metabolic diseases (Vieira de Almeida et al., 2003; Funchal et al., 2005a).

Intermediate filaments are cell-specific proteins forming the cytoskeletal framework in the cytoplasm of various eukaryotic cells (Coulombe and Wong, 2004). In this context, neurofilaments (NF), the IFs of neurons are composed of three different polypeptides whose approximate molecular weights are 200, 160, and 68 kDa, and are commonly referred to as heavy (NF-H), medium (NF-M) and light (NF-L) neurofilament subunits (Ackerley et al., 2000). Glial fibrillary acidic protein is the IF of mature astrocytes (Eng et al., 2000) and vimentin is the IF of cells of mesenchymal origin (Alberts et al., 2002).

The dynamic properties of IF networks is under the control of protein kinases and phosphatases (Izawa and Inagaki, 2006) acting in potential phosphorylation sites located at the amino- and carboxy-terminal tail domains of NF subunits (Nixon and Sihag, 1991). The phosphorylation sites located on the amino-terminal domain of the neurofilament subunits are phosphorylated by second messenger-dependent protein kinases including protein kinase C (PKC), cyclic AMP- (PKA) and Ca^{2+}/calmodulin-dependent (PKCaMII) protein kinases (Sihag and Nixon, 1990). The functional role of neurofilament phosphorylation is to date not completely clear. However, the regulation of IF polymerization by amino-terminal phosphorylation is well described in the literature (Inagaki et al., 1990; Hashimoto et al., 1998). On the other hand, the carboxy-terminal side arm domains of NF-H and NF-M subunits are extensively phosphorylated by several protein kinases, such as glycogen synthetase kinase (GSK) 3, extracellular signal-regulated kinase (ERK), stress activated protein kinase, protein kinase K, PKC and Cdk-5 (Hisanaga et al., 1993; Shetty et al., 1993; Guidato et al., 1996; Sun et al., 1996). It has been demonstrated that *in vitro* phosphorylation of carboxy-terminal domains of NF-H and NF-M straightens individual neurofilaments and promotes their alignment into bundles (Leterrier et al., 1996), whereas *in vivo* phosphorylation of these proteins is associated with an increased interneurofilament spacing (Hsieh et al., 1994). As a consequence, NF-H and NF-M COOH-terminal side arms extend and form crossbridges among neurofilaments and other cytoskeletal elements (Gotow et al., 1994).

It is well established that elevated Hcy levels has been implicated as a risk factor for vascular disease modulating the expression of proteins involved in actin cytoskeleton reorganization as well as the formation and/or maintenance of cell–cell junction (Dardik et al., 2002). Although some evidences pointing that Hcy induces hyperphosphorylation of cytoskeletal proteins in the brain, such as the microtubule-associated protein tau (Ho et al., 2002; Sontag et al., 2007), the actions of Hcy on IF phosphorylation in neural cells are presently unknown. Thus, the aim of the present investigation was to study the *in vitro* effects of Hcy, at a concentration found in hyperhomocysteinemia, on the endogenous IF-associated phosphorylating system and the signaling pathways involved in such effect in slices of hippocampus and cerebral cortex of rats during development.

2. Experimental procedures

2.1. Radiochemical and compounds

$[^{32}P]Na_2HPO_4$ was purchased from CNEN, São Paulo, Brazil, $[^3H]$ cyclic AMP (23 Ci/mmol) was from Amersham International (UK). $[^{45}Ca]CaCl_2$ (sp.act. 321 KBq/mg Ca^{2+}) was purchased from IPEN, São Paulo, Brazil. Optiphase Hisafe III biodegradable liquid scintillation were obtained from PerkinElmer (Boston, USA). D-L Homocysteine, 1,2-bis (2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis (acetoxymethyl ester) (BAPTA-AM), D-2-amino-5-phosphonopentanoic acid (DL-AP5), verapamil hydrochloride, dantrolene, benzamidine, leupeptin, antipain, pepstatin, chymostatin, acrylamide and bis-acrylamide were obtained from Sigma (St. Louis, MO, USA). KN-93, PD98059, U73122, Ly 294002, pertussis toxin were obtained from Calbiochem (La Jolla, CA, USA). All other chemicals were of analytical grade.

2.2. Animals

Wistar rats (9, 12, 17, 21 and 29 days of age) from the Central Animal House of the Department of Biochemistry, ICBS, UFRGS had free access to water and a 20% (w/w) protein commercial chow. They were kept in a room with a 12:12 h light/dark cycle and temperature of 22 ± 1 °C. The "Principles of Laboratory Animal Care" (NIH publication no. 85–23, revised 1985) were followed in all the experiments and the experimental protocol was approved by the Ethics Committee for Animal Research of the Federal University of Rio Grande do Sul, Porto Alegre.

2.3. Preincubation

Rats were killed by decapitation, the cerebral cortex and hippocampus were dissected onto Petri dishes placed on ice and cut into 400 μm thick slices with a McIlwain chopper. Tissue slices were initially preincubated at 30 °C for 20 min in a Krebs–Hepes medium containing 124 mM NaCl, 4 mM KCl, 1.2 mM $MgSO_4$, 25 mM Na–Hepes (pH 7.4), 12 mM glucose, 1 mM $CaCl_2$, and the following protease inhibitors: 1 mM benzamidine, 0.1 μM leupeptin, 0.7 μM antipain, 0.7 μM pepstatin and 0.7 μM chymostatin in the presence or absence of 10 μM KN93, Ly 294002, U73122, 1 μM staurosporin, 200 ng/ml pertussis toxin, 30 μM PD98059, verapamil, dantrolene, 50 μM BAPTA-AM, 100 μM DL-AP5 when indicated.

2.4. Incubation

After preincubation, the medium was changed and incubation was carried out at 30 °C with 100 μl of the basic medium containing 80 μCi of $[^{32}P]$

orthophosphate with or without addition of BAPTA-AM, DL-AP5, verapamil, dantrolene, KN 93, pertussis toxin, PD98059, Ly 294002 or U73122 in the presence or absence of Hcy when indicated. The labeling reaction was normally allowed to proceed for 30 min at 30 °C and stopped with 1 ml of cold stop buffer (150 mM NaF, 5 mM, EDTA, 5 mM EGTA, 50 mM Tris–HCl, pH 6.5, and the protease inhibitors described above). Slices were then washed twice with stop buffer to remove excess radioactivity.

2.5. Preparation of the high salt-Triton-insoluble cytoskeletal fraction from tissue slices

After treatment, preparations of IF-enriched cytoskeletal fractions were obtained from cerebral cortex and hippocampus of rats as described by Funchal et al. (2003). Briefly, after the labelling reaction, slices were homogenized in 400 μl of ice-cold high salt buffer containing 5 mM KH_2PO_4, (pH 7.1), 600 mM KCl, 10 mM $MgCl_2$, 2 mM EGTA, 1 mM EDTA, 1% Triton X-100 and the protease inhibitors described above. The homogenate was centrifuged at 15,800 × *g* for 10 min at 4 °C, in an Eppendorf centrifuge, the supernatant discarded and the pellet homogenized with the same volume of the high salt medium. The resuspended homogenate was centrifuged as described and the supernatant was discarded. The Triton-insoluble IF-enriched pellet, containing NF subunits and GFAP, was dissolved in 1% SDS and protein concentration was determined. The protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

2.6. Polyacrylamide gel electrophoresis (SDS-PAGE)

The cytoskeletal fraction was prepared as described above. Equal protein concentrations were loaded onto 10% polyacrylamide gels and analyzed by SDS-PAGE according to the discontinuous system of Laemmli (1970). After drying, the gels were exposed to X-ray films (X-Omat XK1) at −70 °C with intensifying screens and finally the autoradiograph was obtained. Cytoskeletal proteins were quantified by scanning the films with a Hewlett-Packard Scanjet 6100C scanner and determining optical densities with an Optiquant version 02.00 software (Packard Instrument Company). Density values were obtained for the studied proteins.

2.7. Measurement of cyclic AMP levels

The cyclic AMP levels were measured as described (Tasca et al., 1995). Slices were preincubated in 500 μl of Krebs–Hepes buffer, pH 7.4, at 37 °C for 60 min, the Krebs–Hepes buffer was changed twice during this period. Incubation was then started by adding 100 μM Hcy during 5 min. Incubation was stopped by placing the tubes in an ice-cold bath and samples were processed as previously described. In brief, incubation medium was replaced by 0.5 M perchloric acid, slices were homogenized and an aliquot was used for protein determination. The homogenate was centrifuged (12,800 × *g* for 2 min) and the supernatant was neutralized with 2 M KOH and 1 M Tris/HCl. The pellet was removed by centrifugation (15,800 × *g* for 3 min) and an aliquot from the supernatant was evaporated under a stream of air in a 50 °C bath according to Baba et al. (1982) modified. Residues were dissolved in 50 mM Tris–HCl, pH 7.4, containing 4 mM EDTA. Cyclic AMP content was measured by the protein binding method of Tovey et al. (1974), using $[^3H]$ cyclic-AMP (23 Ci/mmol) and protein kinase A as the binding protein. Radioactivity was counted by liquid scintillation.

2.8. Calcium uptake

$^{45}Ca^2$ uptake was carried out essentially as described by Ichida et al. (1982) with some modifications. Briefly, hippocampal slices were preincubated at 32 °C for 15 min in an incubation medium containing (mM) 127 NaCl; 1.2 $Na2HPO_4{\cdot}7H_2O$; 5.36 KCl; 0.44 KH_2PO_4; 0.95 $MgCl_2$; 0.7 $CaCl_2$; 10 glucose and 0.5 Hepes (pH 7.4). After that, the medium was changed by fresh medium with 0.68 μCi/mL $^{45}Ca^{2+}$ and tissue slices were preincubated during 10 min. After that, tissue slices were incubated for a further 1 min in the absence or the presence of 100 μM Hcy. Controls did not contain Hcy in the incubation medium. After incubation, the slices were washed twice with ice-cold incubation medium with $CaCl_2$ replaced by 10 mM lanthanium ($LaNO_3$). Immediately after washing, 0.3 ml of 0.5 M NaOH was added and the slices were digested

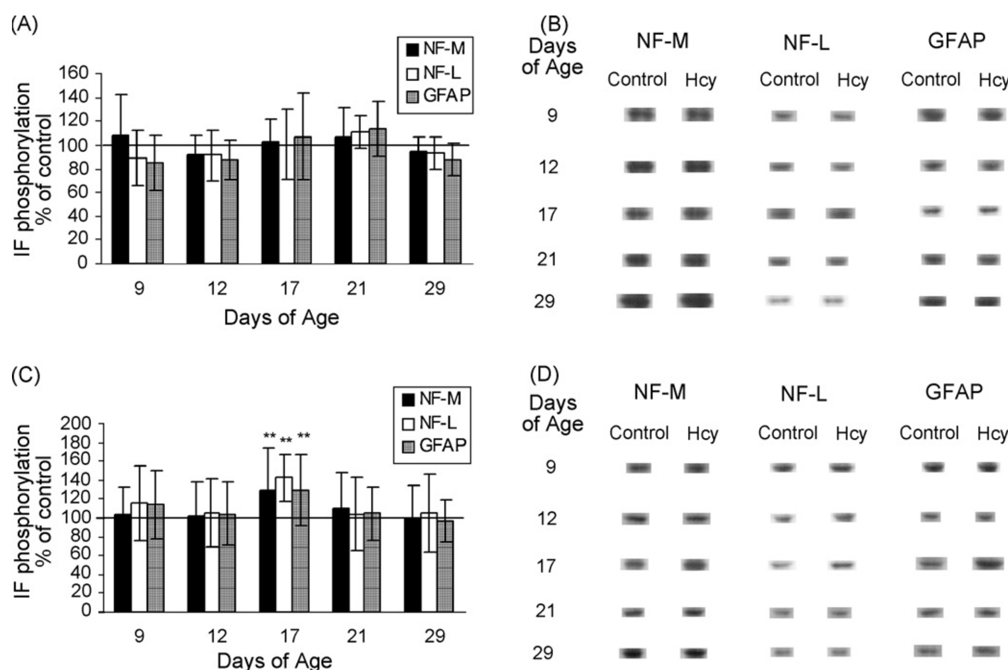

Fig. 1. Effect of Hcy on the *in vitro* phosphorylation of neurofilament subunits and glial fibrillary acidic protein of the cerebral cortex (A and B) and hippocampal (C and D) slices of 9, 12, 17, 21 and 29-day-old rats. Slices of cerebral cortex and hippocampus were incubated with 100 μM Hcy in the presence of ^{32}P-orthophosphate. The cytoskeletal fraction was extracted and the radioactivity incorporated into middle molecular weight neurofilament subunit (NF-M), low molecular weight neurofilament subunit (NF-L) and glial fibrillary acidic protein (GFAP) was measured as described in Section 2. Data are reported as means ± S.D. of 15–20 animals in each group and expressed as percent of controls. Statistically significant differences from controls, as determined by one-way ANOVA followed by LSD test, are indicated: $^{**}P < 0.01$. Representative autoradiographs of cerebral cortex (B) and hippocampus (D) IF phosphorylation.

overnight. Aliquots of the lysates were taken for protein measurement. The remaining lysate was used for determination of the intracellular amount of calcium by liquid scintillation counting. Nonspecific calcium uptake was determined by carrying out the same experiment using a solution containing the nonspecific voltage-dependent calcium blocker La (10 mM). Specific uptake was considered as the difference between total and nonspecific uptake. Radioactivity incorporated was determined with a Wallac Scintillation spectrometer.

2.9. Statistical analysis

Data were analyzed statistically by Student's *t*-test or one-way analysis of variance (ANOVA) followed by the least square difference (LSD) test when the *F*-test was significant. All analyses were performed using the SPSS software program on an IBM-PC compatible computer.

3. Results

We have initially studied the effect of 100 μM Hcy on the *in vitro* phosphorylation of IF-enriched cytoskeletal fraction from cerebral cortex and hippocampus of 9, 12, 17, 21 and 29-day-old rats. Results showed that Hcy was not able to alter the phosphorylation of NF subunits (NF-M and NF-L) and GFAP of cerebral cortex in the ages studied (Fig. 1A and B). On the other hand, Hcy-induced IF hyperphosphorylation in hippocampus from 17-day-old animals (Fig. 1C and D).

Taking into account the importance of Ca^{2+} in a plethora of intracellular events that result in regulation of cell function, particularly the cytoskeletal roles (Stull, 2001), we examined the involvement of Ca^{2+} in the Hcy-mediated increase of the *in vitro* ^{32}P incorporation into IF of 17-day-old rat hippocampus. We first showed that 100 μM Hcy increased Ca^{2+} uptake in tissue slices (Fig. 2A). In order to verify the involvement of Ca^{2+} influx through voltage-dependent calcium channels (VDCC) on Hcy-induced IF hyperphopshorylation, we used the specific L-calcium channel (L-VDCC) blocker verapamil in hippocampus slices. Results showed that coincubation of the slices with 100 μM Hcy plus 30 μM verapamil totally prevented the effect of Hcy on the phosphorylating system, suggesting that Ca^{2+} influx via VDCC was involved in the ability of Hcy to alter the phosphorylating/dephosphorylating equilibrium of IF proteins recovered in the cytoskeletal fraction (Fig. 2B). Next, we tested the influence of an ionotropic glutamate receptor antagonist on the effect of Hcy. Tissue slices were coincubated with 100 μM Hcy and 100 μM DL-AP5, a competitive NMDA ionotropic antagonist. Results showed that hyperphosphorylation induced by Hcy was totally prevented by the NMDA antagonist (Fig. 2B). To further investigate the role of intracellular Ca^{2+} in this process we performed experiments using the membrane-permeable form of BAPTA, namely BAPTA-AM, an intracellular Ca^{2+} chelator, and dantrolene, an inhibitor of ryanodine Ca^{2+} channels. Hippocampal slices were coincubated with 100 μM Hcy plus 50 μM BAPTA-AM, or 30 μM dantrolene. Results showed that both BAPTA and dantrolene prevented the IF hyperphosphorylation induced by Hcy in rat hyppocampus (Fig. 2B).

We also investigated the involvement of PKCaMII, PKC and MAPK in the effect of Hcy on the cytoskeleton by using 10 μM KN93, 1 μM staurosporine and 30 μM PD 98059, the specific kinase inhibitors of PKCaMII, PKC and MAPK, respectively. Results showed that all the inhibitors tested prevented the effect of Hcy on the IF-associated phosphorylating system (Fig. 3A).

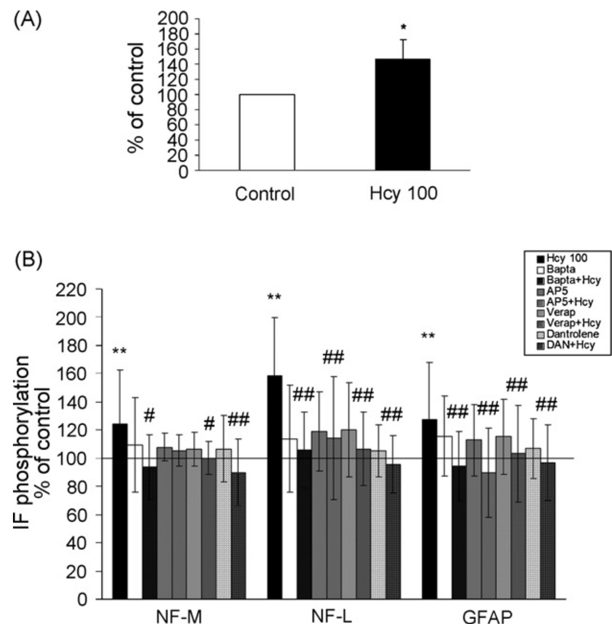

Fig. 2. Effect of calcium on the action of Hyc in hippocampus of 17-day-old rats. (A) Effect of Hcy on $^{45}Ca^{2+}$ uptake in hippocampus of rats. Slices of hippocampus were incubated with 0.7 mM $^{45}Ca^{2+}$ (0.68 μCi) in the presence of 100 μM Hcy. Results are expressed as means ± S.E.M. of 8–12 animals in each group and expressed as percent of control. $^{*}p < 0.01$, different from control (Student's *t*-test). (B) Involvement of intra and extracellular Ca^{2+} on Hcy-induced IF *in vitro* phosphorylation. Hippocampal slices were preincubated for 20 min in the presence or absence of 50 μM BAPTA-AM, 100 μM DL-AP5, 30 μM verapamil, 30 μM dantrolene and incubated with ^{32}P-orthophosphate with or without the blockers and/or 100 μM Hcy. The cytoskeletal fraction was extracted and the radioactivity incorporated into NF-M, NF-L and GFAP was measured. Data are reported as means ± S.D. of 10–15 animals in each group and expressed as percent of control. Statistically significant differences as determined by one-way ANOVA followed by LSD test are indicated: $^{**}P < 0.01$ compared with control group; $^{\#}P < 0.05$ and $^{\#\#}P < 0.001$ compared with Hcy group.

Interestingly, we observed that 5 min incubation of tissue slices with 100 μM Hcy was able to reduce cAMP levels and this effect was prevented by pertussis toxin (200 ng/ml), a G inhibitory (G_i) inhibitor (Fig. 4). To define the possible role of a G_i protein-coupled mechanism in the action of Hcy on IF phosphorylation, tissue slices were treated with this inhibitor in the presence of Hcy. Results showed an inhibition of the Hcy effect by pertussis toxin (Fig. 3B). The contribution of PI_3K and PLC to the Hcy effect was verified by using 10 μM both Ly294002, a PI_3K inhibitor, and U73122, an inhibitor of PLC activity. Results showed that coincubation of Hcy with each inhibitor was able to prevent the effect of the metabolite on IF phosphorylation, evidencing a PI_3K- and PLC-dependent mechanism (Fig. 3B).

4. Discussion

Homocysteine is known to be toxic to the CNS enhancing the vulnerability of neuronal cells to excitotoxic, apoptotic, and oxidative injury *in vitro* and *in vivo* (Maler et al., 2003; Obeid

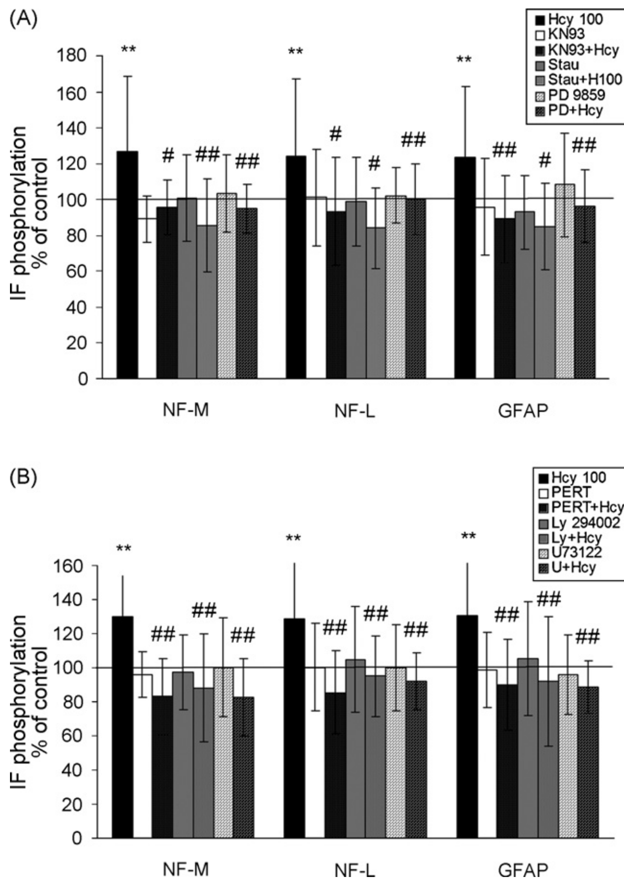

Fig. 3. Involvement of signaling pathways on Hcy-induced IF *in vitro* hyperphosphorylation in hippocampus of 17-day-old rats. (A) Participation of PKCaMII, PKC, MAPK was tested by using the specific inhibitors 10 μM KN93, 1 μM staurosporine, 30 μM PD9859, respectively. (B) Participation of G inhibitory protein, PI3K and PLC on Hcy-induced IF hyperphosphorylation was tested by using the specific inhibitors 200 ng/ml pertusssis toxin (PERT), 10 μM Ly294002 and 10 μM U73122. Hippocampal slices were preincubated in the presence or absence of inhibitors for 20 min and incubated with ^{32}P-orthophosphate with or without 100 μM Hcy and/or inhibitors, as described in Section 2. The cytoskeletal fraction was extracted and the radioactivity incorporated NF-M, NF-L and GFAP was measured. Data are reported as means ± S.D. of 10–15 animals in each group and expressed as % of control. Statistically significant differences from controls, as determined by one-way ANOVA followed by LSD test are indicated: $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with control group; $^{\#}P < 0.05$ and $^{\#\#}P < 0.001$ compared with Hcy group.

and Herrmann, 2006). Moreover, Hcy is able to produce increased intracellular Ca^{2+} levels through NMDA receptors and Erk 1/2 activation in the hippocampus slices of CBS deficient mice (Robert et al., 2005). However no data have yet described the effects of Hcy, at concentrations known to be neurotoxic, on the endogenous phosphorylating system associated with the cytoskeleton, nor the signaling mechanisms involved in such effect in brain slices during development. Taking into account these findings, in the present work we first analyzed the effect of 100 μM Hcy, a plasma concentration found in mild homocystinuric patients, on the phosphorylating system associated with the IF cytoskeleton in slices of cerebral cortex and hippocampus of rats during development. Days 9, 12, 17, 21 and 29 were chosen as representative ages of rat brain

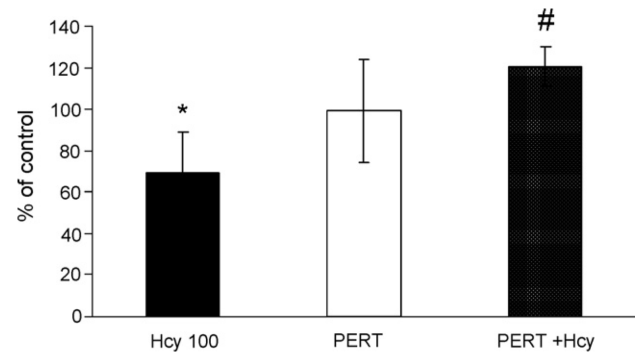

Fig. 4. Effect Hcy on cAMP levels in hippocampus of 17-day-old rats. Tissue slices were incubated for 5 min with 100 μM Hcy and/or 200 ng/ml pertussis toxin (PERT) as described in Section 2. Results were calculated as pmol cAMP/mg protein and expressed as % of control for 10–12 animals in each group. Data are reported as means ± S.D. Statistically significant differences, as determined by one-way ANOVA followed by Tukey–Kramer multiple comparison test, are indicated: $^{*}P < 0.01$ compared with control group; $^{\#}P < 0.001$ compared with Hcy group.

development, according to our previous reports (Vieira de Almeida et al., 2003; Matté et al., 2004). We observed that Hcy was unable to affect the IF phosphorylation of cerebral cortex in the ages studied but induced hyperphosphorylation of neuronal (NF-M and NF-L) and glial (GFAP) IF proteins in the hippocampus of 17-day-old rats, reflecting an altered activity of the endogenous phosphorylating system associated with the IF protein in this cerebral structure. These findings provide an interesting insight on the differential susceptibility of cortical and hippocampal IF cytoskeleton to the *in vitro* exposure to the metabolite and could reflect the existence of a window of vulnerability of the cytoskeleton of hippocampal cells at this developmental age. In this context, it is assumed that various parts of the brain develop at different times and have different windows of vulnerability, both prenatally and postnatally, based on the temporal and regional maturation mediated through a multitude of developmental processes (Rice and Barone Jr, 2000). The effect of Hcy in hippocampal slices, is consistent with previous evidences of Hcy-induced excitotoxicity in rat hippocampus (Kim et al., 2007; Baydas et al., 2005; Robert et al., 2004, 2005; Kruman et al., 2000). Moreover, neurofilament hyperphosphorylation is known to impairs axonal transport leading to aberrant perikaryal or proximal axonal accumulation (Yabe et al., 2000), supporting the importance of Hcy inducing neuronal trauma.

We have chosen to study the involvement of Ca^{2+}-mediated mechanisms on the effects of Hcy on the IF cytoskeleton because of previous evidences showing that Hcy toxicity in the CNS is related to increased cytosolic Ca^{2+} (Lipton et al., 1997; Ho et al., 2003). In this context, the rise in intracellular Ca^{2+} concentrations in response to a stimulus could originate from a Ca^{2+} influx pathway, from release of Ca^{2+} from an internal store, or through a combination of these (Peuchen et al., 1996). The increased ^{45}Ca uptake we evidenced in hippocampal slices treated with 100 μM Hcy pointed to an effect on calcium influx. We then proceeded to the blockage of VDCC and NMDA receptors, known to be important mechanisms of calcium influx (Davare and Hell, 2003; Putney Jr, 1999). In our approach we

used verapamil, a specific L-VDCC blocker and DL-AP5 a competitive NMDA inhibitor. Results showed that in the presence of each blocker, Hcy was not able to exert any effect on IF phosphorylation, suggesting the involvement of VDCC (Niikura et al., 2004) and NMDA (Ozawa et al., 1998) receptors on this action. The evidence of NMDA receptor mediating the action of Hcy on the cytoskeleton is in agreement with Robert et al. (2005) who described Hcy as a NMDA agonist in hippocampal slices of rats. Next, we investigated the role of intracellular Ca^{2+} mobilization in this process using BAPTA-AM, showing that when the intracellular Ca^{2+} was chelated, the effect of Hcy on IF phosphorylation was totally prevented. We also raised the possibility that Ca^{2+} capacitative release from the endoplasmic reticulum through ryanodine receptors could participate in the sequence of events elicited by Hcy as a consequence of the increased extracellular Ca^{2+} influx. This hypothesis was tested using dantrolene which also prevented IF hyperphosphorylation. Taken together these data, we demonstrate that the stimulatory action of 100 μM Hcy on the IF-associated phosphorylating system in the cytoskeletal fraction of 17-day-old rat hippocampus is related to changes in Ca^{2+} influx and mobilization of intracellular Ca^{2+} pools.

Therefore, we identified kinases that could be involved in the Hcy-induced IF hyperphosphorylation. Results indicated that such effect was mediated by two Ca^{2+}-dependent protein kinases, PKCaMII and PKC, as evidenced by using specific protein kinase inhibitors KN-93 (Tokumitsu et al., 1990) and staurosporin (Gescher, 1998), respectively. This is consistent with the increased Ca^{2+} levels and with our previous reports demonstrating that PKCaMII is associated with the cytoskeletal fraction (Funchal et al., 2005b). On the other hand, PKC is an important signal transducing kinase whose activation is dependent on diacylglicerol (DAG) in different cell types (Wang, 2006). Supporting the participation of PKC, we demonstrated the involvement of PLC in Hcy action by using U73122, suggesting that the activation of PKC depends on the phosphatidylinositol hydrolysis by PLC producing inositol 1,4,5-triphosphate (IP_3) and DAG. Production of IP_3 raises $[Ca^{2+}]_i$ by liberating Ca^{2+} from endoplasmic reticulum (ER) stores (Nestler and Duman, 2006), contributing to activation of PKCaMII. Otherwise DAG is an endogenous PKC activator. Our results are in agreement with previous evidences that activation of PKC may induce neurofilament phosphorylation in motor neurons by acting in cooperation with stimulation of NMDA receptors and activation of CaMK. These mechanisms may be relevant to the neuronal injuries induced by Hcy (Doroudchi and Durham, 1997).

Several evidences in the literature point to MAP kinases, particularly Erk1/2, playing a dominant role in phosphorylation of NF proteins (Grant and Pant, 2000). The involvement of MAPK activation on Hcy-stimulated IF phosphorylation was demonstrated by treating tissue slices with PD 98059, a MEK inhibitor, since the effect of Hcy was totally prevented by this drug. These findings are in agreement with previous evidences of Ho et al. (2002), showing that PD 98059 prevented Hcy-induced tau hyperphosphorylation in murine cortical neurons. Moreover, PKC activity has been shown to directly activate Raf-1 (Kolch et al., 1993; Marais et al., 1998) and MEK in hippocampal neurones (Roberson et al., 1999) a step that is upstream in the MAPK cascade (Raf-1/MEK/MAPK). The prevention of Hcy action through inhibition of PKC and MEK is consistent with an effect at the level of Raf-1, meaning that Raf-1 may be phosphorylated by PKC in the Hcy-induced modulation of the cytoskeleton.

In addition, we verified that Hcy was unable to induce IF hyperphosphorylation in the presence of the PI3K inhibitor, Ly 294002. PI3Ks convert phosphatidylinositol 4,5-bisphosphate into phosphatidylinositol 3,4,5-trisphosphate (PIP3). The production of PIP3 by PI3K induces a myriad of cellular responses in neurons, such as transcription (Brunet et al., 2001), translation (Akama and McEwen, 2003), protein trafficking (Lhuillier and Dryer, 2002; Man et al., 2003; Wang et al., 2003), and cytoskeletal rearrangements (Atwal et al., 2003). These responses are mediated by a variety of downstream effectors, including protein kinases phosphoinositide-dependent kinase-1 and Akt, (Jaworski et al., 2005). The PI3K–Akt pathway has been implicated in neuronal growth, survival, neurite outgrowth, and synaptic plasticity (Atwal et al., 2000; Kuruvilla et al., 2000; Markus et al., 2002; Sanna et al., 2002). Then it is feasible that the effects of Hcy on the phosphorylating system associated with the IF proteins was also mediated by a downstream PI3K pathway.

We also demonstrated that mechanisms dependent on the activation of adenylyl cyclase signaling pathway were not implicated in the effect of Hcy. This finding was first supported by the reduction of cAMP levels elicited by Hcy, constituting an intriguing point, since we have previously described increased Ca^{2+}/calmodulin- (PKCaMII) and cAMP- (PKA)-dependent protein kinases activities, in IF hyperphosphorylation in animal models of neurodegenerative diseases (Oliveira-Loureiro et al., 2005; Funchal et al., 2005a,b). In addition, we demonstrated that pertussis toxin prevented the Hcy effect on the cAMP levels, as well as on the IF-associated phosphorylating system, suggesting that the effect of Hcy on the cytoskeleton could be also associated with a G_i protein-coupled receptor (GPCR) at plasma membrane.

Although the exact mechanisms by which Hcy stimulate hippocampal IF phosphorylation are not known, we could propose an initial activation of a G_i protein-coupled receptor at the cell membrane causing reduced cAMP levels and PKA activity. This action would be somehow followed by activation of PLC, PKC and MAPK. This is consistent with Robert et al. (2005) that showed the effects of Hcy on ERK cascade activation were also dependent on Ca^{2+} influx, CaMK-II and PKC activation. We also raise the possibility that IP_3 and DAG could participate in the sequence of events elicited by Hcy, considering the involvement of intracellular Ca^{2+} levels and PKC activation in such effects. Furthermore, Hcy might activate NMDA and voltage-dependent Ca^{2+} channels and the increased intracellular Ca^{2+} levels could induce CICR through ryanodine receptors contributing to activate PKCaMII. The actions of Hcy modulating intracellular Ca^{2+} levels and kinase cascades, summarized in Fig. 5, can represent, at least in part, a mechanism for Hcy targeting

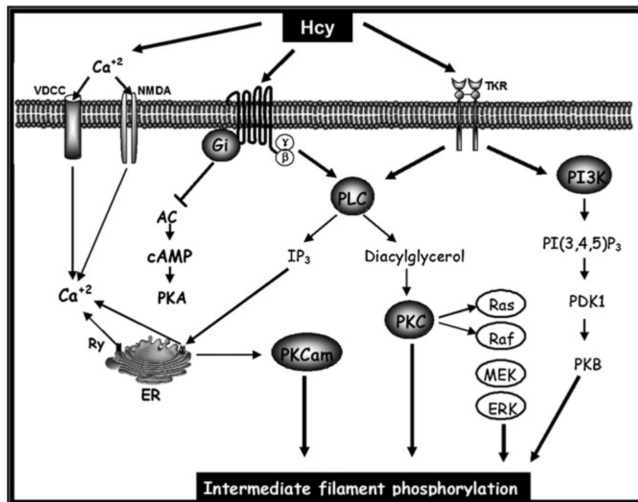

Fig. 5. Proposed mechanism by which Hcy activates IF phosphorylation in hippocampus of rats. Hcy could stimulate G_i protein-coupled, tyrosine kinase, VDCC or NMDA receptors at the cell membrane causing reduced cAMP levels and PKA activity; activation of PLC and PI3K pathways [PI(3,4,5)P_3, PDK1, PKB]; and/or Ca^{2+} influx, respectively. IP_3 and DAG could increase intracellular Ca^{2+} levels and activate PKC, respectively. PKC, for this time, could activate the MAPK cascade (Ras, Raf, MEK, ERK). The increased intracellular Ca^{2+} levels could still induce CICR through ryanodine receptors contributing to PKCaMII activation. VDCC, voltage-dependent Ca^{2+} channel; NMDA, *N*-methyl-D-aspartate receptor; G_i, G inhibitory protein-coupled receptor; TKR, tyrosine kinase receptor; PLC, phospholipase C; PI3K, phosphoinositol-3-kinase; PKCaM, Ca^{2+}/calmodulin-dependent protein kinase; PKC, protein kinase C; ER, endoplasmic reticulum; Ry, ryanodine receptors.

the cytoskeleton, contributing to neurodegeneration associated with hyperhomocysteinemia.

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Capítulo 3

HOMOCYSTEINE INDUCES CYTOSKELETAL REMODELING, APOPTOTIC CELL DEATH AND REACTIVE OXYGEN SPECIES PRODUCTION IN CULTURED CORTICAL ASTROCYTES

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HOMOCYSTEINE INDUCES CYTOSKELETAL REMODELING, APOPTOTIC CELL DEATH AND REACTIVE OXYGEN SPECIES PRODUCTION IN CULTURED CORTICAL ASTROCYTES

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Running title: Homocysteine induces astrocyte cytoskeletal remodeling

Keywords: homocysteine; cytoskeleton; actin; glial fibrillary acidic protein; oxidative stress

ABSTRACT

In this study we treated cortical astrocytes and neurons in culture with homocysteine (Hcy) at different concentrations (10, 100 and 500 µM) and analyzed cytoskeletal remodeling by immunocytochemistry after 24 h exposure. We observed dramatically altered actin and GFAP cytoskeleton exposed to 100 µM Hcy. Induction of reactive oxygen species (ROS), in cultured astrocytes showed a fluctuating production of ROS along 24 h exposure to 10, 100 and 500 µM Hcy. Cytoskeletal remodeling induced by 100 µM Hcy was prevented by the antioxidants folate (5µM) or trolox (80µM). Otherwise, 500 µM Hcy decreased astrocyte proliferation and induced apoptosis after 24 h exposure. Moreover, immunocytochemical analysis showed that 10, 100 or 500 µM Hcy-treated neurons presented unaltered cytoskeletal organization and neuronal death. These findings indicate that the cytoskeleton of cortical astrocytes, but not neurons, in culture are somehow involved in the deleterious actions of Hcy on the CNS, and such effects are mediated by redox signaling mechanisms. Cells were able to respond to mild Hcy concentrations reorganizing their cytoskeleton and surviving, while elevated Hcy impaired cytoskeleleton remodeling leading to apoptotic cell death and these effects might have important implications for understanding the actions of Hcy in the neurotoxicity linked to neurodegenerative disorders.

INTRODUCTION

Astrocytes, the most abundant cells in the central nervous system, provide neurotrophic factors for neurons, control extracellular pH, potassium and glutamate levels and participate in the formation and preservation of the blood brain barrier (Danbolt 2001; Fields and Stevens-Graham 2002).

Cell morphology is principally defined by the cytoskeleton constituents, such as microfilaments, microtubules, and intermediate filaments (IF). However, actin cytoskeleton is the major determinant of cell shape, being involved in cell motility, phagocytosis, migration, adhesion, cytokinesis, and endocytosis (Zigmond 1996). There is considerable evidence that drugs which prevent actin polymerization are able to alter various astrocytic functions (Cotrina et al. 1998; Duan et al. 1999; Lascola et al. 1998; Sergeeva et al. 2000). Central to actin roles is its ability to be reorganized into distinct structures that are suitable for particular tasks at the cell cortex. Evidence from many organisms has implicated changes to the dynamics of the actin cytoskeleton, in the release of reactive oxygen species (ROS) from mitochondria and subsequent cell death (Gourlay and Ayscough 2005). On the other hand, IFs constitute a family of cytoskeletal proteins organized into a three-dimensional scaffold in the cytoplasm. Glial fibrillary acidic protein (GFAP) is the IF present in mature astrocytes, although its precise contributions to astroglial physiology and function are not yet clear. The upregulation of GFAP following injury and astrogliosis has been a long-standing pathological observation (Maragakis and Rothstein 2006) and in vitro studies suggest that the significance of GFAP is magnified following injury in which cellular stability might be affect (Eng et al. 1998; Lipton et al. 1997; Maragakis and Rothstein 2006; Pekny 2001; Wilhelmsson et al. 2004).

Homocystinuria, in inborn errors of metabolism, may lead to hyperhomocysteinemia in the range of 50–500 mM (Lipton et al. 1997; Mudd 2001; Scott and Weir 1998; Yudkoff 2006). Patients with homocystinuria display neurological abnormalities (Bleich et al. 2003; Bleich et al. 2000; Grieco 1977; Levine et al. 2002; Mudd et al. 1985; Sachdev et al. 2002; van den Berg et al. 1995). In recent years, the mechanisms of Hcy-induced damage have been explored in different cell types and tissues (Cattaneo 2003; Cattaneo 2006; Hron et al. 2007; Kruman et al. 2000; Langmeier et al. 2003; Matte et al. 2004; Streck et al. 2004; Streck et al. 2003a; Streck et al. 2003b; Streck et al. 2002; Wyse et al. 2002). In this context, cell damage induced by high homocysteine (Hcy) levels markedly enhances the vulnerability of neuronal cells to excitotoxic and oxidative injury in vitro and in vivo (Kruman et al. 2000; Lipton et al. 1997; Loscalzo 2002; Matte et al. 2008; Mattson and Shea 2003). Recently Maler et al. (2003) have shown that 2 mM Hcy induces cell death of astrocytes in vitro. The oxidative stress-mediated actions of Hcy have been also described to induce cytoskeletal remodeling in endothelial cells (Sen et al. 2007). Earlier reports (Baydas et al. 2006; Baydas et al. 2005), described that Hcy can sensitize glial cells causing toxicity through free radical generation and apoptosis.

We have previously described that Hcy, at a concentration found in hyperhomocysteinemia, acted on the endogenous IF-associated phosphorylating system and such effects were dependent on glutamate receptors and Ca^{2+} channels, activating different signaling pathways in slices of hippocampus and cerebral cortex of rats during development (Loureiro et al. 2008). However, data about the specific effects of Hcy on neuron and astrocyte cytoskeletal organization were lacking. Thus, the aim of the present work was to study the effects of different concentrations of Hcy on the cytoskeletal remodeling of cultured neurons and astrocytes from cerebral cortex of rats, investigating the possible involvement of oxidative stress in this action.

MATERIALS AND METHODS

Materials

D-L homocysteine, creatine, trolox, phalloidin-fluorescein isothiocynate, anti-β tubulin III, anti GFAP (clone G-A-5), monoclonal anti-β-actin, anti-vinculin, anti-mouse IgG (whole molecule)-FITC, F (ab')$_2$ fragment-Cy3, peroxidase conjugated anti-mouse IgG, peroxidase conjugated anti-rabbit IgG and material for cell culture were obtained from Sigma (St. Louis, MO, USA). Polyclonal anti-GFAP was from DAKO and anti-β tubulin III from Promega Corporation. ECL kit was from Amersham, Oakville, Ontario. 4', 6'-diamidino-2-phenylindole (DAPI) was from Calbiochem (La Jolla, CA, USA). Fetal bovine serum (FBS), Neurobasal medium, Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12), fungizone and penicillin/streptomicin were purchased from Gibco BRL (Carlsbad, CA, USA). TUNEL assay Kit (Terminal Deoxynucleotide Transferase dUTP Nick End Labeling A23210, Molecular Probes). TUNEL Assay kit and 2'–7'-dichlorofluorescein diacetate (DCF-DA) was provided from Molecular Probes. All other chemicals were of analytical grade.

Primary astrocyte and neuron cultures

Astrocyte primary cultures were prepared from newborn (0–1-day old; P0) Wistar rats cerebral cortices, as described previously (Trentin and Moura Neto 1995). Dissociated cells were plated in Dulbecco's modified Eagle's medium (DMEM)/F12/10% FBS (pH 7.4) supplemented with glucose (33 mM), glutamine (2 mM), and sodium bicarbonate (3 mM) into a 15.5 mm diameter well (24-well plate) (Corning Inc., New York, New York), previously coated with polyornithine (1.5 mg/ml, Mr 41,000; Sigma, St. Louis, MO). After astrocytes reached semi-confluence, the culture medium was removed by suction and the cells were incubated until 24 hours at 37° C in an atmosphere of CO2/95% air in DMEM F12

without FBS in the presence or absence (controls) of the Hcy concentrations (10, 100 and 500 μM). In experiments using antioxidants (folic acid 5 μM, trolox 80 μM and creatine 5 mM), the astrocytes were pre-incubated with these substances 6 hours before the Hcy treatment.

Primary neuronal cell cultures were prepared from 18 day embryonic (E18). Wistar rats cerebral cortices as previously described (Moura Neto et al. 1983). Briefly, single-cell suspensions were obtained by dissociating cells of cerebral cortex in DMEM/F12 medium supplemented with glucose (33 mM), glutamine (2 mM), and sodium bicarbonate (3 mM). Approximately 5x104 cells were plated either on polyornithine-treated coverslips placed on a 24-well plate to single neuronal cultures. The neuronal cultures were kept in Neurobasal medium supplemented with glutamine (2 mM) for up to 24 h.

Immunocytochemistry

Immunocytochemistry was performed as described previously (Gomes et al. 1999). Briefly, cultured cells plated on glass coverslips were fixed with 4% paraformaldehyde for 30 min and permeabilized with 0.1% Triton X-100 in phosphate-buffered saline (PBS) for 5 min at room temperature. After blocking with BSA 5%/PBS, cells were incubated overnight with polyclonal anti-GFAP (1:500) or -β tubulin III (1:200) at room temperature, followed by PBS washes and incubation with specific secondary antibody conjugated with Cy3 (sheep anti-rabbit; 1:5000) or with fluorescein isothyocyanate (sheep anti-mouse; 1:400) for 1 h. In all immunostaining-negative controls, reactions were performed by omitting the primary antibody. No reactivity was observed when the primary antibody was absent. The actin cytoskeleton was observed using phalloidin-fluorescein isothiocynate and the nucleus was stained with DAPI. Cells were viewed with a Nikon inverted microscope and images transferred to a computer with a digital camera (Sound Vision Inc., USA).

Morphometric analysis

Astrocytes immunostained for actin cytoskeleton and nucleus were measured using the labeling for DAPI and phalloidin-fluorescein. Measurements of cytoplasm and nucleus were obtained using the Image J Software (NIH, Bethesda, MD) and cytoplasm/nucleus ratio of the cells was used as a criterion of morphologic alteration. Astrocytes immunostained for GFAP cytoskeleton were analysed counting the proportion of citoplasmatic and fibrous astrocytes.

The length of the neurites of each cell immunostained for β tubulin III was analyzed using the Image J Software. At least, four independent experiments were performed in triplicate, encompassing ten fields randomly chosen in each group.

Viability studies in cortical neuronal cells

a) Ethidium homodimer assay - For viability studies in neuronal cultures, 40 minutes before completing treatment with Hcy, cells were incubated with ethidium homodimer at 37ºC in an atmosphere of CO_2/95% air to detect dead cells. After, the cells were washed with PBS, fixed with 4% paraformaldehyde and nuclei were stained with DAPI. Cells were viewed with a Nikon inverted microscope and images transferred to a computer with a digital camera. The total quantity of cells was determined with DAPI and dead cells stained with ethidium homodimer. At least four independent experiments were performed in triplicate, encompassing ten fields randomly chosen in each group.

b)Tetrazolium salt method (MTT assay) - The viability assay also was performed by the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. Three hours before completing treatment with Hcy, 0.5 mg/ml of MTT was added to the medium of incubation. The formazan product generated during the incubation was

solubilized in dimethyl sulfoxide (DMSO) and measured at 490 and 630 nm. Only viable cells are able to reduce MTT.

Viability of cortical astrocytes in culture

a) TUNEL assay - Labeling of fragmented DNA ends from apoptotic primary astrocyte cultures was performed by TUNEL assay Kit, following manufacturer's instructions. Briefly, astrocyte cells were seeded onto a 6-well plate culture and treated with 10, 100 and 500 µM Hcy for 24 hours until cells were detached with trypsin/EDTA and suspended in PBS following cell fixation with paraformaldehyde 1% for 15 minutes at 4ºC. After washing (PBS), fixed cells were maintained in ethanol 70% at -20ºC. Blockade was made with 5% BSA for 30 minutes at room temperature. After washing (PBS), cells were incubated with DNA labeling solution for 90 minutes at 37ºC. After, Rinse Buffer washes, cells were then incubated with monoclonal antibody anti-BrdU Alexa Fluor 488 (1:20) for 30 minutes at room temperature. Cells were analysed by flow cytometry (50.000 events per condition) within 3 hours of completing the staining procedure.

b) 3H-thymidine incorporation assay - Semi-confluent monolayers of astrocytes were treated for 24 h with Hcy. 3H-thymidine (0.25 Ci/mL culture medium) was added during the last 6 h of treatment. Newly synthesized DNA was precipitated with 10% TCA, and quantified by liquid scintillation spectrometry, as previously described (Freeman et al. 1996).

Evaluation of intracellular ROS production

Intracellular Reactive Oxygen Species (ROS) production was detected using the nonfluorescent cell permeating compound, 2'–7'-dichlorofluorescein diacetate (DCF-DA). DCF-DA is hydrolyzed by intracellular esterases and then oxidized by ROS to a fluorescent

compound 2'–7'-dichlorofluorescein (DCF). Astrocytes were treated with DCF-DA (10 µM) for 30 min at 37oC. Following DCF-DA exposure, the cells were rinsed and then scraped into PBS with 0.2% Triton X-100. The fluorescence was measured in a plate reader (Spectra Max GEMINI XPS, Molecular Devices, USA) with excitation at 485 nm and emission at 520 nm.

Western blot analysis

After treatment, astrocytes cells were disrupted by lysis solution containing 2mM EDTA, 50mM Tris-HCl, pH 6.8, 4% SDS. For electrophoresis analysis, samples were dissolved in 25% (v/v) of a solution containing 40% glycerol, 5% mercaptoethanol, 50mM Tris-HCl, pH 6.8, and boiled for 3min.

Total protein homogenate (50 µg of protein) were separated by 10% SDS-PAGE (50µg/lane of total protein) and transferred (Trans-blot SD semidry transfer cell, BioRad) to nitrocellulose membranes for 1 h at 15V in transfer buffer (48mM Trizma, 39mM glycine, 20% methanol, and 0.25% SDS). The blot was then washed for 20min in Tris-buffered saline (TBS) (500mM NaCl, 20mM Trizma, pH 7.5), followed by 2 h incubation in blocking solution (TBS plus 5% defatted dry milk). After incubation, the blot was washed twice for 5min with blocking solution plus 0.05% Tween-20 (T-TBS) and then incubated overnight at 4°C in blocking solution containing one of the following monoclonal antibodies: anti-glial fibrillary acidic protein (clone G-A-5) diluted 1:500, anti-β-actin diluted 1:500 and anti-vinculin diluted 1:500. The blot was then washed twice for 5 min with T-TBS and incubated for 2 h in antibody solution containing peroxidase-conjugated rabbit antimouse IgG diluted 1:2000 or peroxidase-conjugated anti-rabbit IgG diluted 1:2000. The blot was again washed twice for 5min with T-TBS and twice for 5min with TBS. The blot was developed using a chemiluminescence ECL kit.

Statistical analysis

Data from the experiments were analyzed statistically by one-way analysis of variance (ANOVA) followed by the Tukey test when the F-test was significant. Values of $p < 0.05$ were considered to be significant. All analyses were carried out in an IBM compatible PC using the Statistical Package for Social Sciences (SPSS) software.

RESULTS

In order to evaluate the effect of Hcy on cytoskeletal organization of astrocytes in culture, cells were treated with different concentrations, 10, 100 and 500 µM Hcy and analyzed by immunocytochemistry after 24 h exposure using phalloidin-fluorescein to decore actin. Results showed that in basal conditions the actin cytoskeleton is largely distributed into the abundant cytoplasm, consistent with a flat and rounded morphology, characteristic of protoplasmic astrocyte cells. Morphologically, alteration in the actin cytoskeleton was not observed until 12h exposure to 10, 100 or 500 µM Hcy (results not shown). However after 24 h, 10 µM Hcy induced a slight reorganization of actin cytoskeleton, while 100 µM Hcy-treated cells presented dramatic cytoskeletal reorganization, as determined by cytoplasm/nucleus relation, reflected by the appearance of fusiform and/or spreading cells with retracted cytoplasm. Otherwise, neither 10 nor 500 µM Hcy-treated cells presented actin reorganization in the different times observed (Figure 1A and B). Also, western blot analysis showed that neither actin nor vinculin - an actin associated protein - immunocontent were altered by Hcy in the three concentrations studied (Figure 1C and D). Similarly, immunocytochemical analysis using Cy3-labelled anti-GFAP antibody showed that under basal conditions, cells presented intense cytoplasmic immunolabeling to GFAP. In addition, nearly all cultured cells (>98%) stained positively to GFAP, attesting to their astrocyte

phenotype, and in control cells GFAP stained filaments formed a meshwork extending across the cytoplasm. In cells exposed to 10, 100 and 500 µM Hcy for 24 h, we observed that only 100 µM Hcy induced a rearrangement of the GFAP filament network, evidenced by the increased percent of fibrous astrocytes, without altering total immunocontent of this protein (Figure 2A and B). Moreover altered GFAP cytoskeleton was not observed until 12h exposure to 10, 100 or 500 µM Hcy (results not shown). Phalloidin-GFAP co-staining showed the prominent astrocyte stellation of 100 µM Hcy-treated cells (Figure 3).

The antioxidants folate (FA) (5µM) or trolox (80µM), that did not present effect per se, were able to prevent actin remodeling in 100 µM Hcy-treated astrocytes after 24 h exposure. Conversely, creatine (5mM) was ineffective in preventing such effect (Figure 4A and B). Induction of ROS, measured by DCF-DA assay, in cultured astrocytes along 24 h exposure to 10, 100 and 500 µM Hcy, was assayed. Results showed a rapid production of ROS at 1h exposure to 10, 100, or 500 µM Hcy. It is interesting to note that at 1 h exposure ROS production was prominent in 100 µM exposed cells (150%), as compared to the lower and higher Hcy concentrations (40 and 50% respectively). At 6 h treatment, ROS levels remained increased, restoring basal values at 24 h, except for 100 µM Hcy-treated cells, where high ROS levels were persistent until 24 h (Figure 5).

3H-thymidine incorporation was measured to evaluate cell proliferation and results showed that only the higher Hcy concentration (500 µM) decreased the 3H-thymidine incorporation into DNA of cultured astrocytes after 24 h exposure (Figure 6A). Next, we evaluated the ability of the different Hcy concentrations for inducing astrocyte apoptosis, determined by TUNEL assay. We observed that only 500 µM Hcy was able to greatly increase the number of apoptotic cells after 24 h exposure (Figure 6B).

Immunocytochemical analysis using FITC-labeled anti-β tubulin III antibody was carried out to test neuronal cytoskeleton susceptibility to the effects of the different

concentrations of Hcy. Results showed that 10, 100 or 500 μM Hcy-treated cells presented complex neurite meshworks containing long processes, similar to those observed in control cells as determined by measuring neurite lengths (Figure 7A and B). Also, MTT and ethidium homodimer viability assays demonstrated that Hcy at the concentrations studied, was not able to induce cell death in cultured cortical neurons (Figure 7C and D).

DISCUSSION

We have previously demonstrated that Hcy at concentrations found in blood of patients with mild (100 μM) and severe (500 μM) hyperhomocysteinemia respectively, altered the in vitro 32P incorporation into neurofilaments subunits and GFAP from hippocampus slices of rats during development through glutamate receptors- and Ca^{2+}-mediated mechanisms. Interestingly, Hcy was not able to affect the IF-associated phosphorylating system in slices of cerebral cortex of rats (Loureiro et al. 2008). However, in the present report we show that Hcy induced cytoskeletal remodeling and apoptotic cell death in cortical astrocytes in culture, but not in neurons and this effect was dependent on oxidative stress. These findings highlight the complexity and heterogeneity of neural cells throughout the CNS.

We showed that after 24 h exposure, 100 μM Hcy provoked cytoplasm retraction with redistribution of actin filaments without changing actin or vinculin immunocontent, suggesting that Hcy did not alter the amount of total actin or adhesion/focal contacts in these cells. Otherwise, actin remodeling supports morphological alterations in astrocytes, as previously described (Funchal et al. 2004) and suggests a role for the cytoskeleton in cell survival of 100 μM Hcy-treated astrocytes. We also evidenced an altered organization of GFAP filaments in cells treated with 100 μM Hcy for 24 h, supporting therefore the

reorganization of actin cytoskeleton. Our observations indicated that Hcy at a concentration found in mild hyperhomocysteinemia induced a dramatic effect on the cytoskeleton, while these effects were not observed in a Hcy concentration compatible with severe hyperhomocysteinemia (500µM). The reasons leading to the apparent maintenance of cytoskeletal organization of astrocytes exposed to 500 µM Hcy could be ascribed to induction of actin stabilization with reduction of actin turnover. These findings supporting links between actin and apoptosis pathways came from studies using drugs that affect actin turnover (Gourlay and Ayscough 2005; Odaka et al. 2000; Posey and Bierer 1999). Moreover, this is consistent with our data showing apoptotic cell death in astrocytes exposed for 24h to 500 µM Hcy, in contrast with the absence of apoptosis in 100 µM Hcy-treated cells, which presented disorganized cytoskeleton. As regards the consequences of alterations of the actin cytoskeleton network, it may have important implications on various critical astrocyte functions (Cotrina et al. 1998; Duan et al. 1999; Lascola et al. 1998; Sergeeva et al. 2000), including endo and exocytosis of vesicles and protein trafficking (Ridley and Hall 1994). Otherwise astroglial dysfunction, and even dysregulation of astroglial-specific functions are associated with various neurological diseases (Seifert et al. 2006).

It is important to emphasize that cortical neurons in culture were not susceptible to cytoskeletal remodeling induced by the concentrations of Hcy used in our study. This is consistent with a selective vulnerability of cultured neurons from different brain structures exposed to Hcy. Although, at present we do not know the reasons leading to the selective action of Hcy on glial rather than neuronal cytoskeletal reorganization, these findings support an active role of astrocytes to neuronal injury showing that alterations in the partnership between astrocytes and neurons could be important mechanisms underlying brain lesions (Volterra and Meldolesi 2005). The importance of neurons on brain integrity was previously evidenced by Luo et al (2007) who described that injected Hcy into the lateral cerebral

ventricle of rats induced hyperphosphorylation of the axonal microtubule-associated protein tau prominently at 6, 9, and 12 h after the injection, and it was recovered to normal at 24 h, suggesting short-term effects of Hcy followed by long-lasting recovering of neuronal cells.

Oxidative stress has been related to the actions of elevated Hcy in different tissues (Garcia et al. 2008; Kassab et al. 2008; Zhu et al. 2008), including in brain (Ansari and Bhandari 2008). Thus, we could propose that the persistently elevated ROS levels after 24 h exposure to 100 μM Hcy are related with reorganization of actin and GFAP filaments in astrocytes, since Hcy is redox-active, and its toxic effects have been frequently ascribed to redox signaling pathways in vascular (endothelial and smooth muscle) and in neuronal cells (Matte et al. 2004; Streck et al. 2002; Wyse et al. 2002; Zou and Banerjee 2005).

Otherwise, the ability of folate and antioxidant supplementation to reverse or at least modulate the detrimental effects of Hcy in neurodegenerative diseases has been extensively investigated (Ho et al. 2003). Folate deprivation and increased Hcy interact synergistically with, and potentiate, neural insult and this effect is particularly deleterious in developing nervous system (Mattson and Shea 2003). In this context, the neuroprotective roles of folate and trolox (a non-metabolizable form of vitamin E) against the Hcy effects was evidenced by us, since they were able to prevent the action of 100 μM Hcy on actin cytoskeleton in astrocytes.

On the other hand, the system phosphocreatine/CK represents an important mechanism to maintain cellular energy homeostasis of cells with high and fluctuating energy demand, like the neural cells (Wallimann et al. 1992). In order to verify whether Hcy might impair energy production in the brain (Halestrap et al. 1974), we supplemented creatine to the cultures, however the morphological alterations induced by Hcy were not prevented. We concluded that impairment of energy homeostasis is not involved in the action of Hcy on the astrocyte cytoskeleton in culture, despite impairment of brain energy metabolism has been

described in rat brain slices treated with 100 µM Hcy (Streck et al. 2003b). In additon, marked decrease of respiratory chain complex I activity has been described in the cerebral cortex of immature rats treated with dl-homocysteic acid (Folbergrova et al. 2007). These findings emphasize a role for astrocytes as local communication elements of the brain that can generate various regulatory signals and networks that are otherwise disconnected from each other (Volterra and Meldolesi 2005). In this context, the loss of neuron-glia interactions could illustrate the importance of an integrated network of both synaptic and non-synaptic routes supporting the energy homeostasis in brain.

Consistent with previous evidence that folate deficiency and elevated Hcy can damage and kill neurons in cell culture, and increase their vulnerability to being killed by various excitotoxic, oxidative and metabolic insults (for review see (Mattson and Shea 2003)), we assayed cultured astrocytes and neurons to cell death. Our results showed that Hcy at the concentrations used was not able to induce neuronal cell death. Similarly, this metabolite at a concentration able to induce persistently high ROS levels and cytoskeletal remodeling at 24 h exposure, did not affect cell proliferation nor induced apoptotic cell death. On the other hand, the insult of 500 µM Hcy, that failed to induce cytoskeletal reorganization, decreased astrocyte proliferation and induced apoptosis. Thus, increased turnover of filamentous actin could promote cell longevity, whereas decreased actin turnover could trigger cell death through an apoptosis-like pathway. This is consistent with the roles of actin mediating cell responses to both internal and external signals (Gourlay and Ayscough 2005). Conversely, alterations in actin dynamics can both increase and decrease levels of detectable ROS, which has led to the hypothesis that the actin cytoskeleton could function as a regulator for ROS release from the mitochondrion. On the basis on these findings, we could propose that astrocytes exposed to 500µM Hcy lost the ability for remodeling their cytoskeleton and this could be related with modulation of ROS levels. Otherwise, this insult

would be able to elicited nuclear signaling pathways leading some cells to a decreased proliferation and compelling others to the apoptotic process.

Taken together, the present findings indicate that the cytoskeleton of cortical astrocytes in culture, but not neurons, are somehow involved in the deleterious actions of Hcy on the CNS, and such effects are mediated by redox signaling mechanisms. Cells are able to respond to mild Hcy concentrations reorganizing their cytoskeleton and surviving, while elevated Hcy elicits more dramatic effects impairing cytoskeleleton remodeling and leading to apoptotic cell death. Considering that astrocytes are polyvalent cells that are implicated in almost all processes that occur in the CNS, the vulnerability of astrocyte cytoskeleton may have important implications for understanding the effects of Hcy in the neurotoxicity linked to neurodegenerative disorders.

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LEGENDS

Figure 1. Actin cytoskeleton organization of cortical astrocytes exposed to homocysteine (Hcy). Cells were cultured to semi-confluence in DMEM/F12 10% fetal bovine serum (FBS). The medium was then changed to DMEM/F12 0% FBS in the presence or absence of Hcy (10, 100 and 500 µM) for 24h. (A) Representative images of Hcy treated cells immunostained with phalloidin-fluorescein and DAPI nuclear staining. Scale bar = 50 µm. (B) Morphometric analysis from control and treated astrocytes. The cells were analyzed using the ratio between the size of cytoplasm and nucleus with the Image J Software. Data are reported as means ± SEM of five different experiments. Statistically significant difference from controls as determined by ANOVA followed by Tukey test is indicated: * $<P <0.01$. Western blot analysis of actin (C) and vinculin (D) immunocontent in cell homogenate. All lanes received equivalent amount (50µg) of total protein from cell extract. Immunoblotting was carried out with monoclonal anti-β-actin and anti-vinculin antibodies diluted 1:500. The blots were developed using an ECL kit. Data are reported as means ± SEM of four different experiments expressed as percentage of controls. Data were statistically analyzed by one-way ANOVA. Representative immunological reactions are shown.

Figure 2. Effect of Hcy on GFAP organization in cortical astrocytes. Cells were cultured to semi-confluence in DMEM/F12 10% fetal bovine serum (FBS). The medium was then changed to DMEM/F12 0% FBS in the presence or absence of Hcy at different concentrations (10, 100 and 500 µM) for 24h. (A) Representative images of Hcy-exposed cells immunostained for GFAP and DAPI. Scale bar = 50 µm. (B) Western blot analysis of GFAP immunocontent in cell homogenate. All lanes received equivalent amounts (50µg) of total protein from cell extract. Immunoblotting was carried out with monoclonal anti-GFAP

antibody diluted 1:500. The blots were developed using an ECL kit. Data are reported as means ± SEM of four different experiments. Data were statistically analyzed by one-way ANOVA. Representative immunological reactions are shown. (C) Morphometric analysis from control and treated astrocytes. The cells were analyzed counting the number of citoplasmatic and fibrous astrocytes immunostained for GFAP from 10 field for each treatment. Data are reported as means ± SEM of five different experiments.

Figure 3 Actin and GFAP co-staining of cortical astrocytes exposed to 100 µM Hcy. Cells were cultured to semi-confluence in DMEM/F12 10% fetal bovine serum (FBS). The medium was then changed to DMEM/F12 0% FBS in the presence or absence of 100 µM Hcy for 24h. Representative images of Hcy treated cells immunostained with phalloidin-fluorescein, GFAP and DAPI nuclear staining. Scale bar = 50 µm.

Figure 4. Effects of folate (FA), trolox (Tro) and creatine (Cre) on Hcy-induced actin cytoskeleton organization in cortical astrocyte. Cells were cultured to semi-confluence in DMEM/F12 10% fetal bovine serum (FBS). The medium was then changed to DMEM/F12 0% FBS and astrocytes were pre-incubated with FA, tro and Cre 6 h before the 100 µM Hcy treatment for 24h. (A) Representative images of treated cells immunostained with phalloidin-fluorescein. Insets show DAPI nuclear staining. Scale bar = 50 µm. (B) Morphometric analysis from control and treated astrocytes. The cells were analyzed using the ratio between the size of cytoplasm and nucleus with the Image J Software. Data are reported as means ± SEM of four different experiments. Statistically significant differences as determined by one-way ANOVA followed by Tukey test are indicated: *P < 0.01 compared with control group; #P < 0.01 compared with Hcy group.

Figure 5. Effect of different concentrations of Hcy on the induction of reactive oxygen species (ROS) in astrocytes. Cells were cultured to confluence in DMEM/F12 10% fetal bovine serum (FBS). The medium was then changed to DMEM/F12 0% FBS in the presence or absence of Hcy (10, 100 and 500 µM) for 1, 6 and 24h. The intracellular ROS levels were measured with DCF-DA. The fluorescence was measured in a fluorescence microplate reader (excitation 485 nm and emission 520 nm). Results are expressed as percent of controls. Statistically significant differences from controls, by one-way ANOVA followed by Tukey's multiple variation test, are indicated: $^*P < 0.01$.

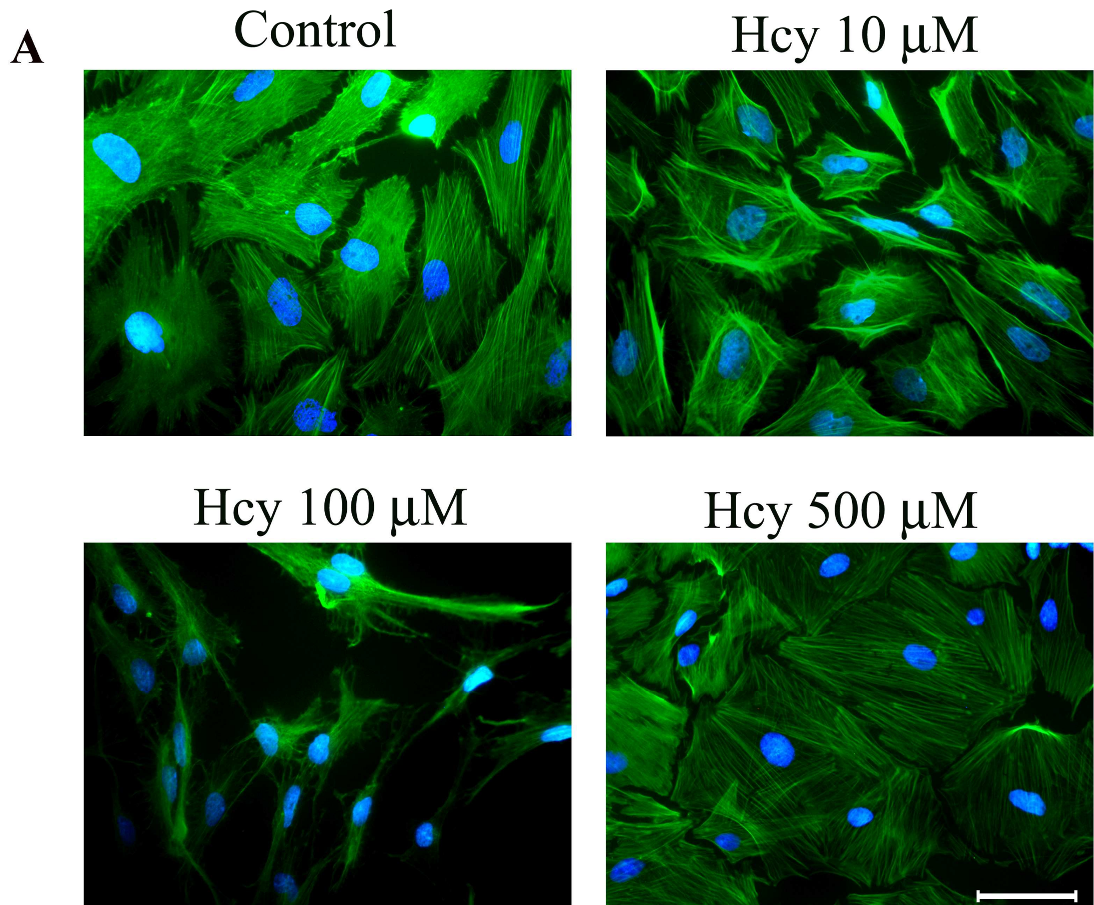
Figure 6. Effect of Hcy on astrocyte viability. Cells were cultured to semi-confluence in DMEM/F12 10% fetal bovine serum (FBS). The medium was then changed to DMEM/F12 0% FBS in the presence or absence of Hcy at different concentrations (10, 100 and 500 µM) for 24h. (A) 3H-thymidine incorporation assay in Hcy-treated astrocytes. 3H-thymidine (0.25 Ci/mL culture medium) was added during the last 6 h of treatment. Basal and Hcy-induced 3H-thymidine incorporation was measured in the presence of DMSO. (B) Labeling of fragmented DNA ends from apoptotic astrocytes was performed by TUNEL assay Kit. Cells were analyzed by flow cytometry (50.000 events per condition). Data are reported as means ± SEM of four different experiments. Statistically significant difference from controls, by one-way ANOVA followed by Tukey's multiple variation test, are indicated: $^*P < 0.01$.

Figure 7. Effect of Hcy on neuronal cytoskeleton and viability. Neuronal cells were cultured in Neurobasal medium supplemented with glutamine for 24 h. After, the cells were treated with Hcy at different concentrations (10, 100 and 500 µM) for 24 h. (A) Representative images of Hcy treated cells immunostained with anti-β tubulin III (green). Insets show ethidium homodimer staining. Scale bar = 50 µm. (B) Analysis of neurite length

of neurons treated with Hcy. The length of the neurites of each cell immunostained for β tubulin III was analyzed using the Image J Software. (C) Viability assay performed by the colorimetric MTT method. Three hours before completing treatment with Hcy, 0.5 mg/ml of MTT was added to the incubation medium. The formazan product generated was measured at 490 and 630 nm. (D) Ethidium homodimer method to detect dead cells was performed in cortical neuron cultures. Forty minutes before completing treatment with Hcy, cells were incubated with ethidium homodimer at 37ºC in an atmosphere of CO2/95% air. Four independent experiments were performed in triplicate, encompassing ten fields randomly chosen on the each group. Four independent experiments were performed in triplicate, encompassing ten fields randomly chosen on each group. Data were statistically analyzed by one-way ANOVA.

FIGURE 1

A



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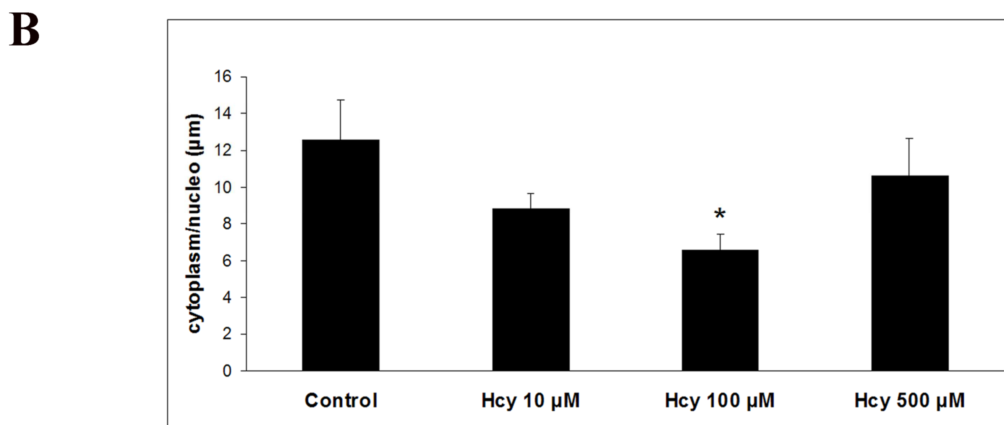

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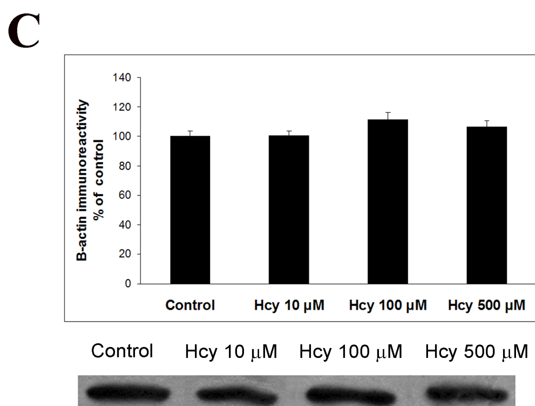

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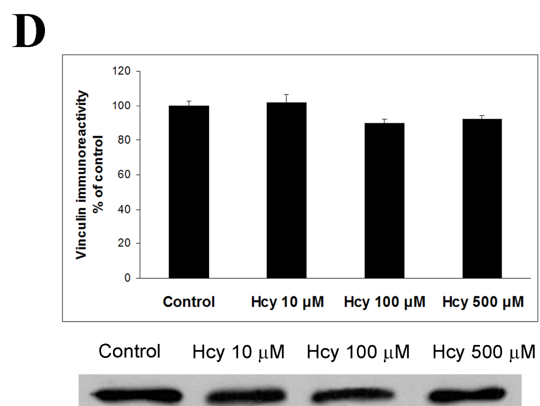


FIGURE 2

A

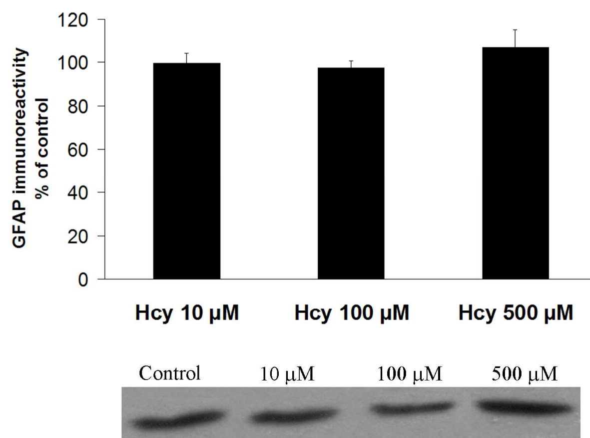
Control

Hcy 10 µM

Hcy 100 µM

Hcy 500 µM

B



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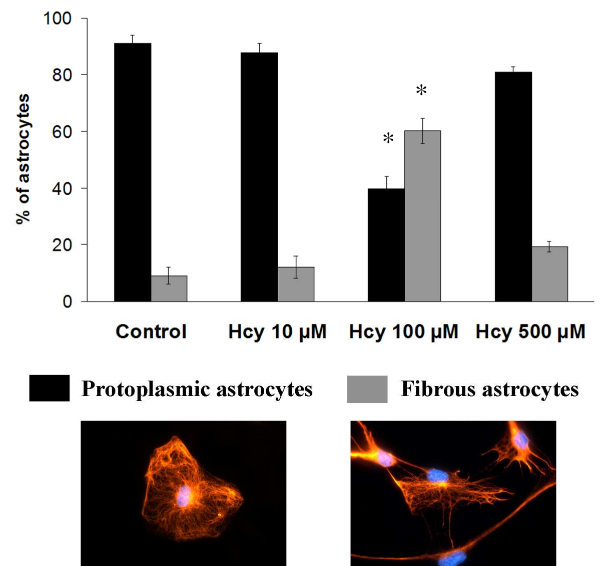
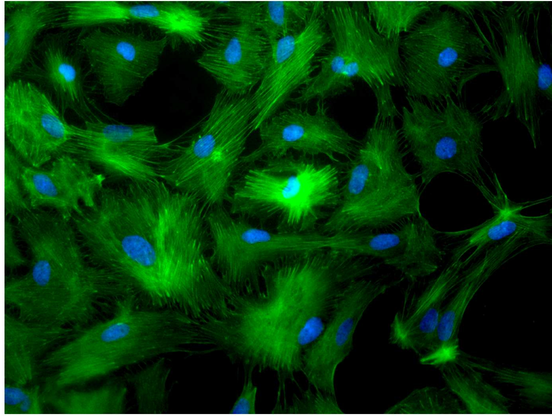
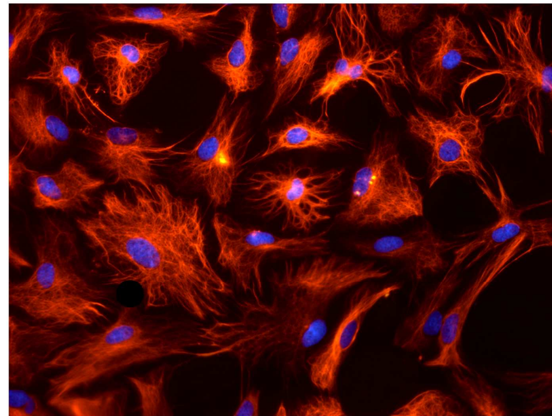
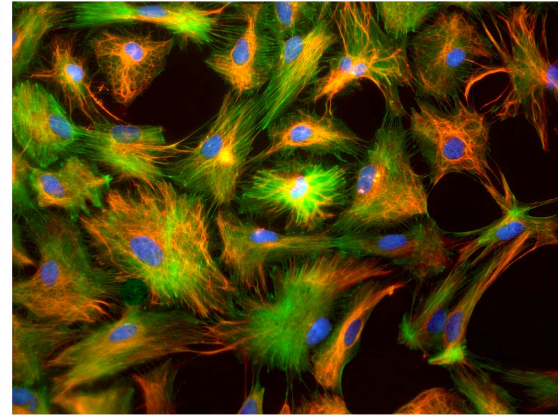
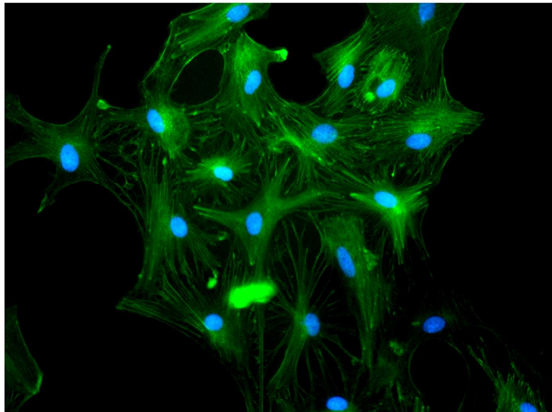
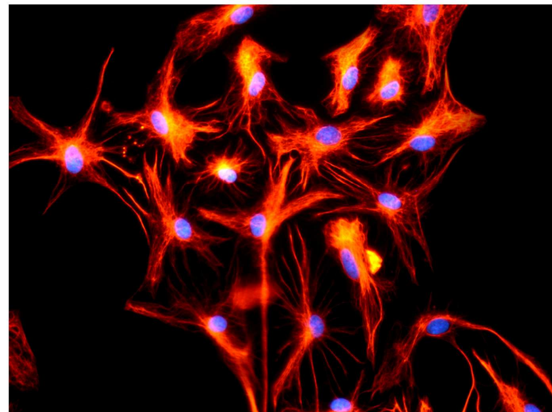
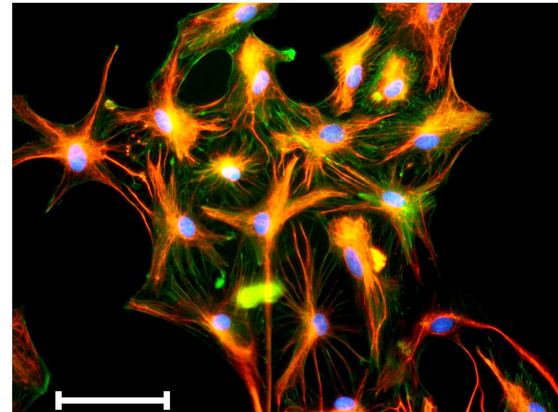


FIGURE 3

Actin / DAPI | GFAP/ DAPI | Merged

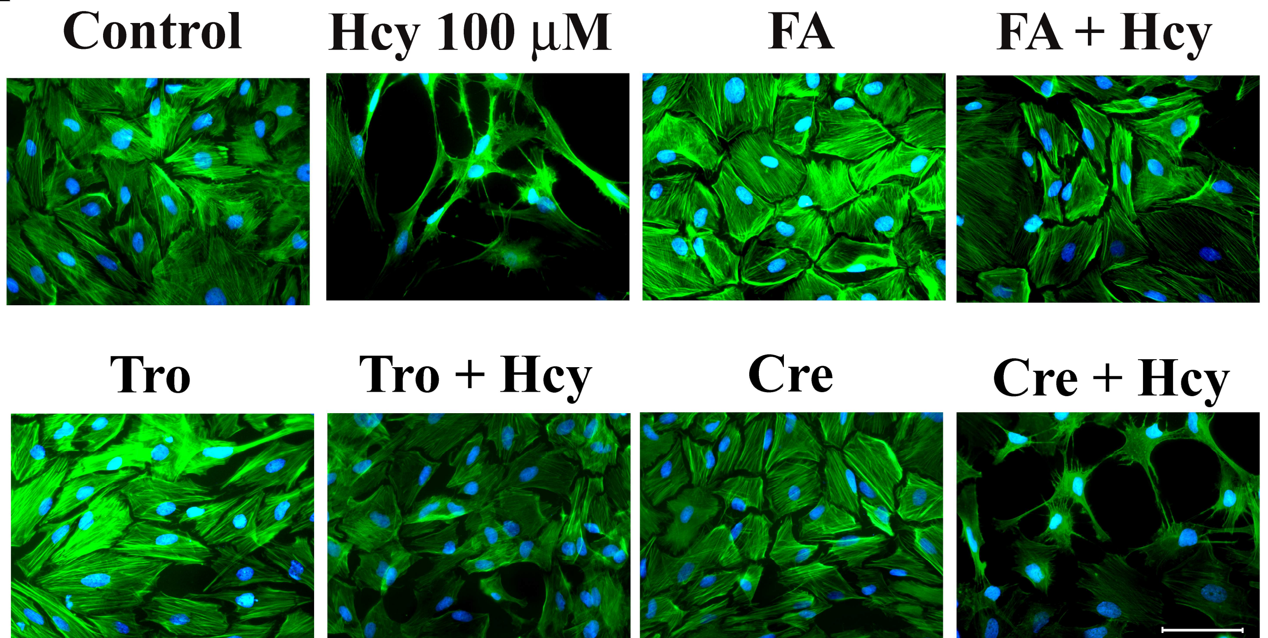
Control

Hcy 100 μM

FIGURE 4

A



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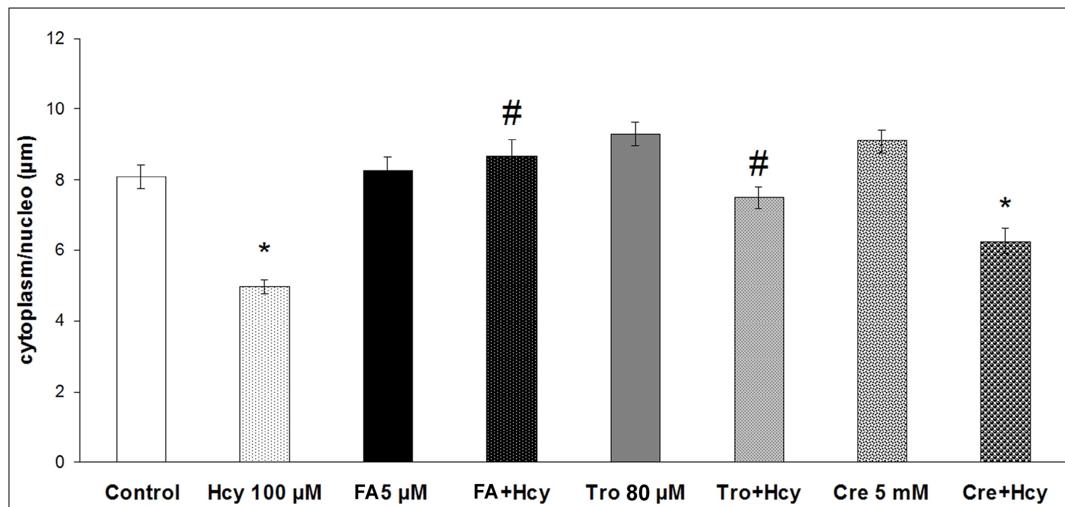


FIGURE 5

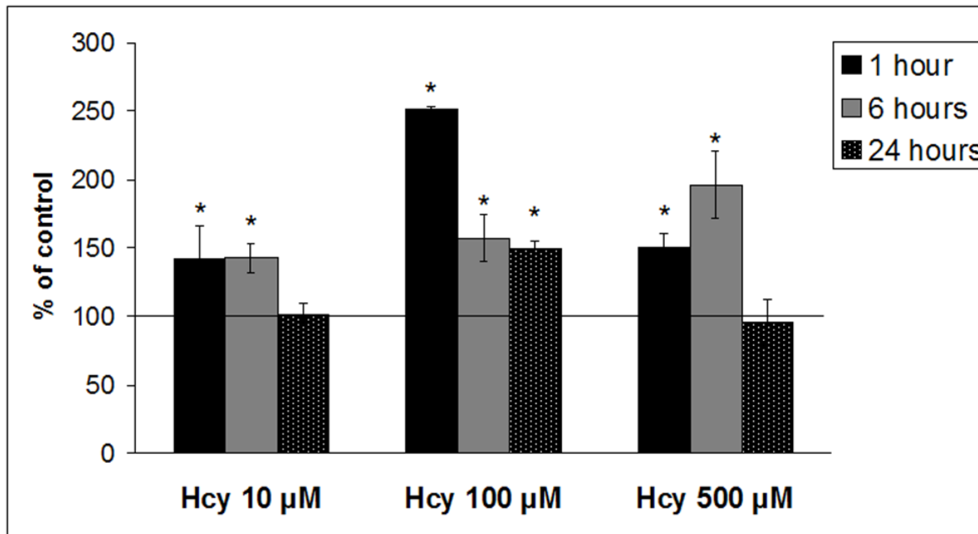
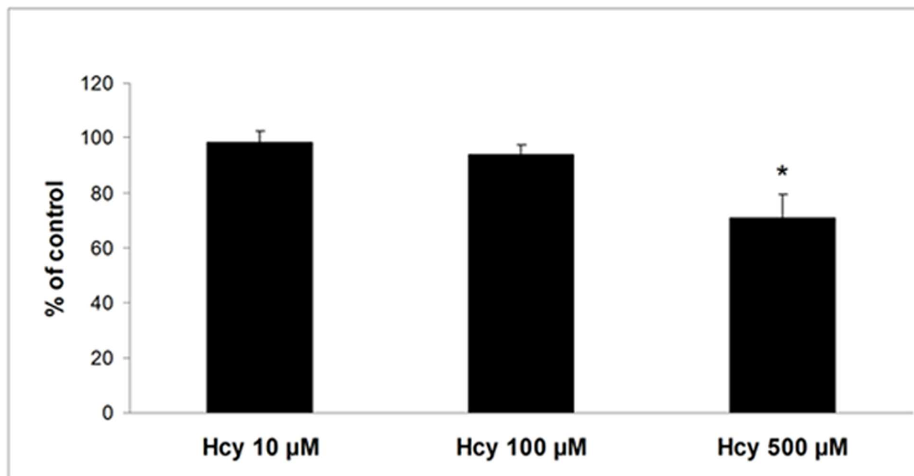


FIGURE 6

A



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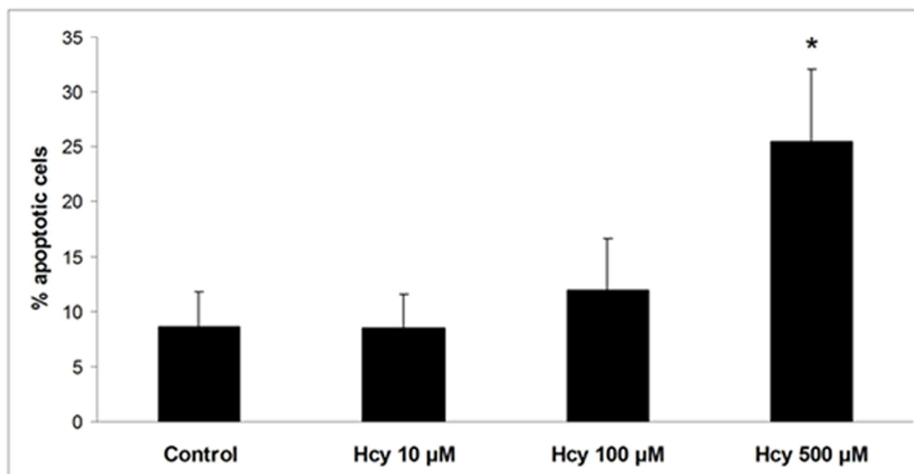
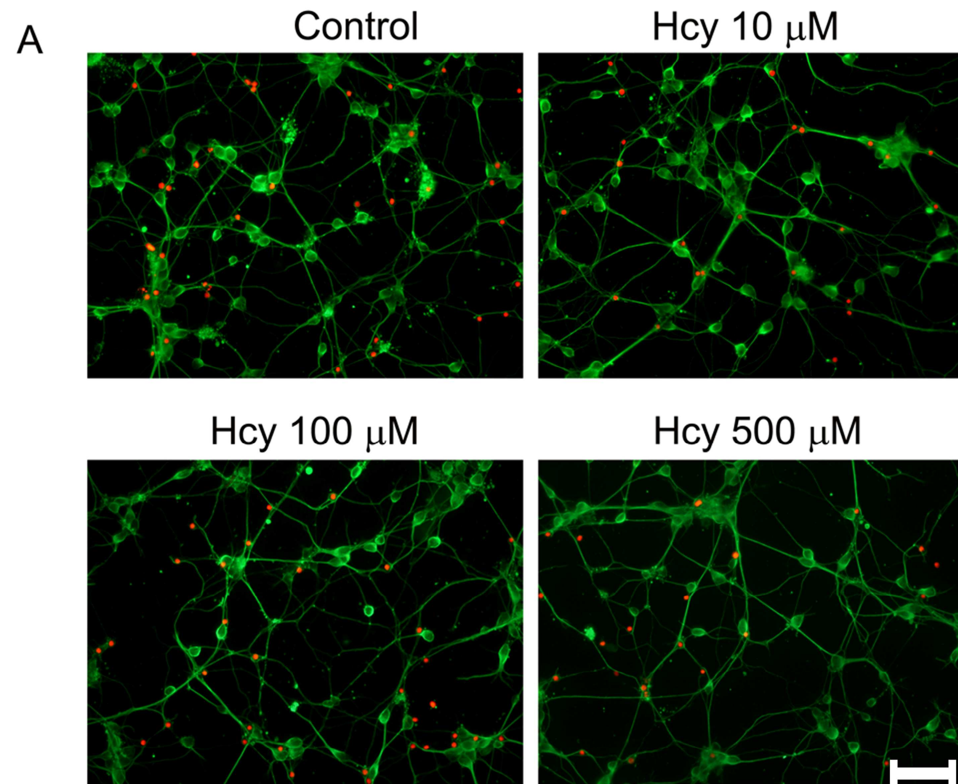
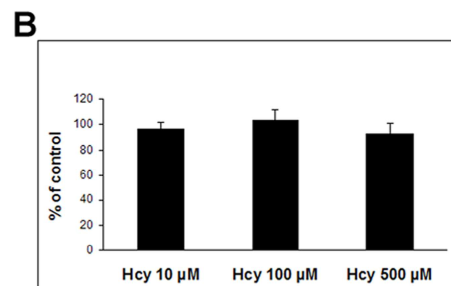
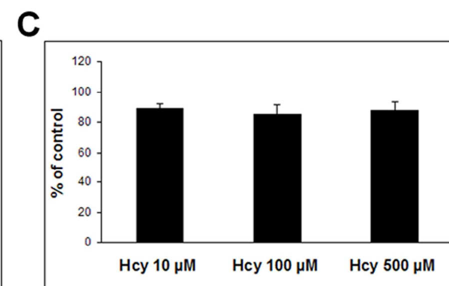
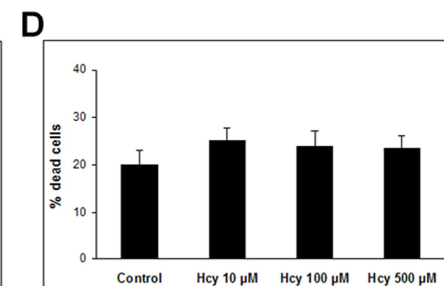


FIGURE 7









Capítulo 4

HOMOCYSTEINE INDUCES HYPOPHOSPHORYLATION OF INTERMEDIATE FILAMENTS AND REORGANIZATION OF ACTIN CYTOSKELETON IN C6 GLIOMA CELLS

Artigo submetido para Cellular and Molecular Neurobiology

HOMOCYSTEINE INDUCES HYPOPHOSPHORYLATION OF INTERMEDIATE FILAMENTS AND REORGANIZATION OF ACTIN CYTOSKELETON IN C6 GLIOMA CELLS

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Running title: Homocysteine and cytoskeleton in C6 cells

SUMMARY

In this study we investigated the actions of high homocysteine (Hcy) levels (100 and 500 μM) on the cytoskeleton of C6 glioma cells. Hcy induced hypophosphorylation of vimentin and glial fibrillary acidic protein (GFAP) in the cytoskeletal fraction. This effect was prevented by the competitive NMDA ionotropic antagonist DL-AP5, the non-selective group I/group II metabotropic antagonist MCPG, and the competitive non-NMDA ionotropic antagonist CNQX. Also, 100 or 500 μM Hcy, dramatically increased the number filopodia. Moreover, vinculin immunocontent was upregulated and cdc42 activation was observed. In cells treated with 100 μM Hcy, folic acid, trolox and ascorbic acid, totally prevented filipodia formation, while filopodia induced by 500 μM Hcy were prevented by ascorbic acid and attenuated by folic acid and trolox. Finally, DL-AP5 totally prevented the formation of filopodia in both 100 and 500 μM Hcy treated cells, while MCPG prevented only the effect of 100 μM Hcy on actin cytoskeleton. In conclusion, our results show that Hcy at different concentrations target the cytoskeleton of C6 cells through glutamate mechanisms and/or oxidative stress. Therefore, we could propose that the dynamic restructuring of the cytoskeleton of glial cells might contribute to the response to the injury provoked by elevated Hcy levels in brain.

Keywords: homocysteine; cytoskeleton; actin; glial fibrillary acidic protein; C6 glioma cells.

INTRODUCTION

The cytoskeleton is responsible for maintaining cell morphology, cell movement and adhesion, cell division, and transmembrane signal transmission (Kasas et al., 2005). Dynamic interactions among the cytoskeletal systems regulate to a great extent the structural organization of the cytoplasm of animal cells (Chang and Goldman, 2004).

Microtubules (MTs) are essential for the survival of all eukaryotes organizing the cytoplasm, positioning the nucleus and organelles and so contributing to cell motility. Microfilaments (MFs) or actin filaments support a myriad of processes ranging from cell motility, division and morphogenesis to intracellular protein trafficking (Bloom et al., 2003; Fifkova and Delay, 1982; Hirokawa et al., 1989; Landis et al., 1988). Remodeling of actin cytoskeleton in response to stress factors is a fundamental process in many eukaryotic cells requiring that cells are able to couple a signaling pathway involved in cell viability to remodeling of the actin cytoskeleton (Gourlay and Ayscough, 2005). A large number of low molecular weight, GTP binding proteins of the Ras superfamily have been identified to have a role in cell motility and cytoskeletal remodeling and among them Rho, Rac and Cdc42 are critically involved in actin reorganization (Aspenstrom, 1999; Ridley, 2001; Takai et al., 2001). In particular, Cdc42 is known to stimulate the extension of filopodia, finger-like structures at the periphery of cells that contain bundles of filamentous actin and link the cells to the substratum (Hall, 1998).

Glial fibrillary acidic protein (GFAP) is the intermediate filament (IF) present in adult astrocytes (Eliasson et al., 1999), however, this protein is also expressed in tumors derived from astrocytic lineage (Rutka et al., 1997). The function of GFAP remains not completely understood even though recent findings suggest GFAP involvement in the long-term maintenance of brain architecture, in the proper function of the blood–brain barrier, and

in the modulation of some neuronal functions by astrocytes (Liedtke et al., 1996; McCall et al., 1996; Pekny et al., 1998; Shibuki et al., 1996). The constituent proteins of IFs are constantly undergoing active exchange between IF polymers and disassembled IF subunits and this balance is regulated by kinase and phosphatase activities. Also, IFs are also involved in the regulation of various signaling events and this regulation is, in turn, controlled by phosphorylation (Hyder et al., 2008; Omary et al., 2006).

The C6 glioma cell (rat glial tumor cell line) has provided the most appropriate cell line for molecular neurobiologists to study sensitivity of glial cells to various substances and conditions, because of its similarities with primary glial cells in culture (Kumar et al., 1984; Vernadakis et al., 1991; Vielkind et al., 1990). With successive passages (beyond passage 70) C6 glioma cell differentiates into a primarily astrocytic cell like population (Parker et al., 1980) and exhibits many properties of astrocytes, including expression of the astrocyte specific markers GFAP (Bissell et al., 1974) and S–100 protein (Pfeiffer et al., 1970). Therefore, the C6 glioma cell line has frequently been used as an in vitro model to study changes in glial cells (Funchal et al., 2005a; Mangoura et al., 1989; Vernadakis et al., 1991).

The nervous system might be particularly sensitive to extracellular homocysteine (Hcy) (Sachdev, 2005). In fact, this molecule promotes excitotoxicity via stimulation of NMDA receptors, group I metabotropic glutamate receptor, increases oxidative stress and damages to the neuronal DNA, thereby triggering apoptosis and excitotoxicity, important mechanisms in neurodegeneration (Ho et al., 2003; Ho et al., 2002; Kruman et al., 2000; Matte et al., 2004; Oldreive and Doherty, 2007; Sachdev, 2005; Streck et al., 2003; Zieminska et al., 2003). Also, high levels of Hcy promote oxidative stress in vascular cells and tissues because of the formation of reactive oxygen species (ROS), through redox-sensitive signaling events (Papatheodorou and Weiss, 2007). Despite the increasing evidence of excitotoxic mechanisms of Hcy, the actions of Hcy on the neural cytoskeleton is poorly

known. In this context, we have recently reported that mild Hcy levels induced IF hyperphosphorylation in rat hippocampus through signaling mechanisms dependent on glutamate receptors and Ca2+ concentrations, suggesting that the cytoskeleton of hippocampus slices is susceptible to neurotoxic concentrations of Hcy during development (Loureiro et al., 2008).

Taking into account the previous evidence in the literature and our recent findings, in the present study we used C6 glioma cells to analyze the ability of Hcy in concentrations found in mild and severe hyperhomocysteinemia (Mudd, 2001), to in reorganize MT, GFAP and MF cytoskeleton. We also focused on some intracellular mechanisms leading to cytoskeletal remodeling in C6 cells. We hypothesize that cytoskeletal remodeling supports cell damage induced by Hcy.

METHODS

Radiochemicals and Compounds

[^{32}P]orthophosphate was purchased from CNEN, São Paulo, Brazil, D-L homocysteine (Hcy), creatine, trolox, benzamidine, leupeptin, antipain, pepstatin, chymostatin, acrylamide, bis-acrylamide, phalloidin-fluorescein isothiocynate, anti GFAP (clone G-A-5), monoclonal anti-α-tubulin clone B-5-1-2, monoclonal anti-β-actin, anti-vinculin, anti-rabbit IgG (whole molecule), F (ab')2 fragment-Cy3, anti-mouse IgG (whole molecule)-FITC, peroxidase conjugated rabbit anti-mouse IgG and material for cell culture were purchased from Sigma (St. Louis, MO, USA). Polyclonal rabbit anti-glial fibrillary acidic protein was obtain from Dako Corp. (CA, USA). Cdc42 activation assay Biochem Kit (BK034) was purchased from Cytoskeleton (Denver, USA). Fetal bovine serum (FBS), Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12), fungizone and

penicillin/streptomicin were purchased from Gibco BRL (Carlsbad, CA, USA). All other chemicals were of analytical grade.

Maintenance of Cell Line

The C6 rat glioma cell line was obtained from Rio de Janeiro Cell Bank (Rio de Janeiro, Brazil). We used C6-glioma cells at passage 80, expressing GFAP and vimentin. The cells were grown and maintained in DMEM/F12/10% FBS (pH 7.4) containing 2.5 mg/ml fungizone, 100 mg/ml penicillin/streptomyicin and supplemented with glucose (33 mM), glutamine (2 mM) and sodium bicarbonate (3 mM). Cells were kept at a temperature of 37◦C, a minimum relative humidity of 95%, and an atmosphere of 5% CO2 in air.

After cells reached semi-confluence, the culture medium was removed by suction and the cells were incubated for 4 h at 37◦C in an atmosphere of 5% CO2/95% air in DMEM/F12 (pH 7.4) containing 0% FBS in the presence or absence (controls) of 100 and 500 μM Hcy. Morphological studies were performed using cells fixed for immunocytochemistry.

Immunocytochemistry

Immunocytochemistry was performed as described previously (Gomes et al., 1999). Briefly, cultured cells plated on glass coverslips were fixed with 4% paraformaldehyde for 30 min and permeabilized with 0.1% Triton X-100 in phosphate-buffered saline (PBS) for 5 min at room temperature. After blocking, cells were incubated overnight with polyclonal anti-GFAP (1:500) our monoclonal anti-α-tubulin (1:500) at room temperature, followed by PBS washes and incubation with specific secondary antibody conjugated with Cy3 (1: 1000) and FITC (1:1000) or with fluorescein isothyocyanate (2g/ml) for 1 h. In all immunostaining-negative controls, reactions were performed by omitting the primary antibody. No reactivity was observed when the primary antibody was excluded. The nucleus was stained with DAPI

(0,25 μg/ml). Cells were viewed with a Nikon inverted microscope and images transferred to a computer with a digital camera (Sound Vision Inc., USA).

In Vitro Phosphorylation

C6 cells were incubated for 3 h in the presence of Hcy, then the medium was changed and incubation for 1 hour was carried out at 30ºC with 1000μL of a medium containing (in mM) NaCl, 124; KCl, 4; MgSO4, 1.2; Na-HEPES, 25; (pH 7.4), glucose, 12; CaCl2,1 and the following protease inhibitors: 1mM benzamidine, 0.1μM leupeptin, 0.7μM antipain, 0.7μM pepstatin, 0.7μM chymostatin, and 10μCi of [32P] orthophosphate with or without addition of the Hcy. In the experiments designed to study signaling mechanisms, cells were previously pre-incubated for 1 hour in the presence or absence of 50 μM DL-AP5, MCPG and CNQX, folic acid (5 μM), ascorbic acid (1 mM), trolox (80 μM). The labelling reaction was normally allowed to proceed for 1 h at 30◦C and stopped with 1mL of cold stop buffer (150mMNaF, 5mM, EDTA, 5mMEGTA, Tris-HCl 50 mM, pH 6.5, and the protease inhibitors described above). Cells were then washed twice by decantation with stop buffer to remove excess radioactivity.

Preparation of the High-Salt Triton Insoluble Cytoskeletal Fraction

After treatment, preparations of total IF were obtained from C6 cells as described by Funchal et al. (2003). Briefly, after the labelling reaction, cells were homogenized in 200μL of ice-cold high salt buffer containing 5mM KH2PO4, (pH 7.1), 600mM KCl, 10mM $MgCl_2$, 2mM EGTA, 1mM EDTA, 1% Triton X-100, and the protease inhibitors described above. The homogenate was centrifuged at 15800 × g for 10min at 4°C, in an Eppendorf centrifuge, the supernatant discarded, and the pellet homogenized with the same volume of the high-salt medium. The resuspended homogenate was centrifuged as described and the supernatant was

discarded. The Triton-insoluble intermediate filament-enriched pellet, containing GFAP and vimentin, was dissolved in 1% SDS and protein concentration was determined by the method of Lowry et al. (1951).

Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The cytoskeletal fraction and the total protein homogenate were prepared as described above. Equal protein concentrations were loaded onto 10% polyacrylamide gels and analyzed by SDS-PAGE according to the discontinuous system of Laemmli (1970). After drying, the gels were exposed to X-ray films (X-Omat XK1) at −70°C with intensifying screens and finally the autoradiograph was obtained. Proteins were quantified by scanning the films with a Hewlett-Packard Scanjet 6100C scanner and determining optical densities with an Optiquant version 02.00 software (Packard Instrument Company). Density values were obtained for the studied proteins.

Total Protein Homogenate

C6 cells were homogenized in 200µL of a lysis solution containing 2mM EDTA, 50mM Tris-HCl, pH 6.8, 4% SDS. For electrophoresis analysis, samples were dissolved in 25% (v/v) of a solution containing 40% glycerol, 5% mercaptoethanol, 50mM Tris-HCl, pH 6.8, and boiled for 3min.

Western Blot Analysis

Cytoskeletal fraction or total protein homogenate were separated by 10% SDS-PAGE (50µg/lane of total protein) and transferred (Trans-blot SDsemidry transfer cell, BioRad) to nitrocellulose membranes for 1 h at 15V in transfer buffer (48mMTrizma, 39 mM glycine, 20% methanol, and 0.25% SDS). The blot was then washed for 10min in Tris-buffered saline

(TBS) (0.5M NaCl, 20mM Trizma, pH 7.5), followed by 2 h incubation in blocking solution (TBS plus 5% defatted dry milk). After incubation, the blot was washed twice for 5min with blocking solution plus 0.05% Tween-20 (T-TBS) and then incubated overnight at 4°C in blocking solution containing one of the following monoclonal antibodies: antiglial fibrillary acidic protein—GFAP (clone G-A-5) diluted 1:500, α-tubulin diluted 1:500 and actin diluted 1:500. The blot was then washed twice for 5min with T-TBS and incubated for 2 h in antibody solution containing peroxidase-conjugated rabbit antimouse IgG diluted 1:2000. The blot was again washed twice for 5min with T-TBS and twice for 5min with TBS. The blot was developed using a chemiluminescence ECL kit (Amersham, Oakville, Ontario).

Evaluation of Intracellular ROS Production

Intracellular Reactive Oxygen Species (ROS) production was detected using the nonfluorescent cell permeating compound, 2'–7'-dichlorofluorescein diacetate (DCF-DA). DCF-DA is hydrolyzed by intracellular esterases and then oxidized by ROS to a fluorescent compound 2'–7'-dichlorofluorescein (DCF). After treatment with Hcy, C6-glioma-cells were treated with DCF-DA (10 μM) for 30 min at 37°C. Following DCF-DA exposure, the cells were rinsed and then scraped into PBS with 0.2% Triton X-100. The fluorescence was measured in a plate reader (Spectra Max GEMINI XPS, Molecular Devices, USA) with excitation at 485 nm and emission at 520 nm.

Rho GTPase Activation Assay

The level of Cdc42-GTP was determined using a assay that measures the formation of Cdc42-GTP based on its specific binding to the GTPase-binding domain of p21-activated kinase (Benard et al., 1999). Target cells (107 cells/ml) were stimulated with Hcy for the times indicated. At the appropriate time, cell activation was stopped by addition of ice-cold

lysis buffer (50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 0.3 M NaCl, 2% IGEPAL). Cell lysates were immediately placed at 4 °C and then clarified by low speed centrifugation at 4°C. Guanine nucleotide loading of lysates and affinity precipitation using GST-p21-binding domain were carried out as described (Benard et al., 1999). The quantity of total protein assayed from the lysate was dermined by the method of Lowry et al. (1951).

Statistical Analyses

Data from the experiments were analyzed statistically by one-way analysis of variance (ANOVA) followed by the Tukey test when the F-test was significant. Values of $p < 0.05$ were considered to be significant. All analyses were carried out in an IBM compatible PC using the Statistical Package for Social Sciences (SPSS) software.

RESULTS

We have initially studied the effects of 100 and 500μM Hcy on the in vitro phosphorylation of vimentin and GFAP present in the IF-enriched cytoskeletal fraction from C6 glioma cells. Figure 1A shows the Coomassie blue stained high salt Triton insoluble cytoskeletal fraction as well as the autoradiograph of the same fraction after the in vitro phosphorylation of the cells with 32P-orthophosphate. Expression of GFAP and vimentin identified by Western blot analysis attest the astrocytic phenotype of the cells. When C6-glioma cells were incubated for 1 or 4 hours with 100 or 500 μM Hcy, the in vitro 32P incorporation into GFAP and vimentin was significantly decreased only after 4 h exposure to the metabolite for both concentrations used (Figure 1B) without affecting vimentin and GFAP immnunocontent in the cytoskeketal fraction (Figure 1C). In order to evaluate the involvement of oxidative stress on the Hcy-induced hypophosphorylation, we co-incubated

C6-glioma cells with Hcy and/or with ascorbic acid (1 mM), trolox (80 μM) and folic acid (5 μM) (Figure 2). Results showed that these compounds did not prevent IF hypophosphorylation caused by Hcy. Next, we examined the involvement of glutamate receptors on this effect and we observed that the competitive NMDA ionotropic antagonist DL-AP5 (50 μM), the non-selective group I/group II metabotropic antagonist MCPG (50 μM), or the competitive non-NMDA ionotropic antagonist CNQX (50 μM), did not presented effect per se, however, they prevented the Hcy-induced IF hypophosphorylation (Figure 3) .

We also investigated the effects of 100 and 500 μM Hcy on the organization of MF, MT and GFAP in C6-glioma cells. Immunocytochemical analysis using anti-GFAP and anti-α-tubulin antibodies showed that 100 or 500 μM Hcy for up to 4 hours was not altered as compared to control cells (Figure 4A and C). Otherwise, immunostaining of cells with phalloidin-fluorescein showed, as expected, that in control conditions actin was organized into filaments distributed throughout the cytoplasm and concentrated near cell membrane. After 4 h exposure to 100 or 500 μM Hcy, the labeling throughout the cytoplasm and staining near the cell membrane remained unaltered. The most remarkable effect was the dramatically increased number of actin stained filopodia observed both in 100 or 500 μM Hcy-treated cells (Figure 4E). In this context, Western blot analysis showed that β-actin, α-tubulin or GFAP immunocontents were not altered (Figure 4B, D and F), after 4h exposure to Hcy in the concentrations studied. On the other hand, vinculin immunocontent was upregulated in extracts of cells treated with 100 or 500 μM Hcy (Figure 5A). Otherwise Cdc42 activation was observed at 5 and 15 min exposure to 100 μM Hcy, while 500 μM Hcy induced a constant Cdc42 activation from 5 to 30 min exposure to the amino acid (Figure 5B). It is important to note that cell viability, evaluated by MTT assay in cells incubated for 1, 4, 12 and 24 h with 100 or 500 μM Hcy showed no significant difference between control and Hcy treated cells (data not show).

Since we have shown that folic acid, vitamins E (trolox) and ascorbic acid prevent behavioral and biochemicals alterations caused by Hcy (Matte et al., 2006; Stefanello et al., 2005; Tagliari et al., 2006), we also tested whether the effect of Hcy on the formation of filopodia in C6-glioma cells could be prevented by these compounds. Cells were treated with folic acid (5μM), trolox (80 μM) and ascorbic acid (1 mM) in the absence (controls) or presence of 100 or 500 μM Hcy for 4 h. We observed that they totally prevented the 100 μM Hcy-induced filopodia formation. Conversely, filopodia induced by 500 μM Hcy were totally prevented by ascorbic acid and attenuated by folic acid and trolox (Figure 6). Based on these results, we also tested the effect of 100 μM Hcy on a measure of oxidative stress namely DCF-DA assay. Results showed increased ROS at 30 min exposure to 100 μM Hcy restoring basal levels after 1h (Figure 7).

Finally, we examined the involvement of glutamate receptors in the Hcy-induced filopodia formation. The NMDA antagonist DL-AP5 (10 μM) totally prevented the formation of filopodia in both 100 and 500 μM Hcy treated cells, while the metabotropic antagonist MCPG (50 μM) prevented only the effect of 100 μM Hcy on actin cytoskeleton. Conversely, CNQX (25 μM) was not able to prevent the effects of Hcy on the reorganization of actin cytoskeleton in the two concentrations used (Figure 8).

DISCUSSION

In the present study we initially observed that both 100 and 500 μM Hcy, concentrations compatible with mild and severe homocysteinemia respectively (Mudd et al., 2001), induced hypophosphorylation of vimentin and GFAP in C6 cells, without altering the immunocontent of these proteins. Aberrant cytoskeletal phosphorylation/dephosphorylation may have serious consequences on cellular function and structure. Therefore, we investigated

whether GFAP hypophosphorylation could affect IF cytoskeletal organization in C6 glioma cells. However, we showed that hypophosphorylation of GFAP and vimentin was not sufficient to alter the organization of GFAP filaments in these cells. The complex phosphorylation patterns of IF indicate that there are specific roles for individual phosphorylation sites that reflect the state of the cell (Ubersax and Ferrell, 2007). Reversible phosphorylation, which has significant roles in regulating the structure and assembly of IF, also serves other functions, such as signaling and cytoskeletal cross-talk (Chang and Goldman, 2004). Although our findings show that IF cytoskeleton is a target to the signaling mechanisms elicited by Hcy in C6 cells, we have no direct evidence to suggest the relationship between IF hypophosphorylation and actin remodeling. In addition, Hcy failed to alter microtubule arrays and tubulin immunocontent, suggesting that in this cell lineage microtubule organization, beyond IFs is resistant to the action of this metabolite at the concentrations used. Otherwise, although 100 and 500 μM Hcy did not alter actin immunocontent, we observed actin remodeling, with formation of filopodia, long unbranched cytoplasm extensions formed by dynamic actin filaments. Although we do not have adequate means to express the filopodia formation quantitatively, the dramatic number of spike protrusions observed in Hcy exposed cells allowed us to ascribe these findings to a significant effect of the treatment. Also, increased vinculin immunocontent in treated cells suggests the formation of attachments of actin filaments to integrins at focal adhesions, which is required for stabilization of filopodia and lamelipodia (Varnum-Finney and Reichardt, 1994). Filopodia are implicated in several fundamental physiological processes, such as cell migration and cell-cell adhesion (Mattila and Lappalainen, 2008). Although the roles of filopodia in glial cells remain to be clarified, Werbowetski and Shivers (2001) have related increased formation of filopodia in C6 glioma cells with cell aggregation and migration throughout a collagen matrix, which could be implicated in the formation of metastasis.

Consistent with the formation of filopodia, the activity of Cdc42 was increased in different patterns in response to 100 and 500 μM Hcy, respectively. Cdc42 is a member of the Rho family of small GTPases, described to control signal transduction pathways linking membrane receptors to the reorganization of the actin cytoskeleton and filopodial extensions at the cell periphery (Hall, 2005). Also, activation of Cdc42 leads to the formation of vinculin-containing focal complexes at the cell periphery and along the tips of growing filopodia (Nobes and Hall, 1995).

Physiological roles of filopodial extensions could allow highly dynamic interactions with the surrounding synapses and the end-feet of the glial-vascular interface, important for signal propagation (Benediktsson et al., 2005; Hirrlinger et al., 2004; Mulligan and MacVicar, 2004; Simard et al., 2003). Thus, misregulation of the physiological equilibrium of cytoskeletal dynamics of glial cells could disrupt the blood-brain barrier resulting in exposure of the brain to near plasma levels of Hcy leading to NMDA mediated excitotoxicity (Lipton et al., 1997). Moreover, our data about the relevance of actin reorganization in response to Hcy are in agreement with previous evidence showing that increased turnover of filamentous actin in response to an insult takes a role in the ability of the cell to respond to this insult and to survive, whereas decreased actin turnover seems to trigger to cell death (Gourlay and Ayscough, 2005). This hypothesis is further supported by the evidence that Hcy at the concentrations used failed to induce cell death.

Homocysteine enhances the vulnerability of neuronal cells to excitotoxic and oxidative injury in vitro and in vivo (Bleich et al., 2002; Ho et al., 2001; Ho et al., 2002; Kruman et al., 2000; Kruman et al., 2002; Lipton et al., 1997; Loscalzo, 2002; Mattson and Shea, 2003; Outinen et al., 1998). In agreement with these findings, in the present study we showed that the action of 100 and 500 μM Hcy on the cytoskeleton of C6 cells was mediated by glutamate receptors and by oxidative stress, reinforcing the cytoskeleton as a target of Hcy

neurotoxicity (Loureiro et al., 2008) and illustrating the complexity of concentration-dependent Hcy actions.

In this context, GFAP and vimentin hypophosphorylation induced by 100 and 500 µM Hcy was prevented by ionotropic and metabotropic glutamate antagonists, however filopodia formation was mediated by NMDA and metabotropic group I/II glutamate receptors but not by non NMDA ionotropic receptors. Also, transiently increased ROS production at 30 min exposure to 100 µM Hcy, suggested that oxidative stress could contribute to the formation of filopodia, since this effect was totally prevented by ascorbic acid and by folic acid. Filopodia formation caused by 500 µM Hcy was partially prevented by these substances, suggesting that oxidative stress is at least in part involved in the effect of Hcy at this concentration. Regarding folic acid, it is important to emphasize that this vitamin exerts an important role over Hcy catabolism, by methyl group donation in remethylation pathway to methionine (Brosnan et al., 2004; Finkelstein, 1998), beyond its antioxidant properties (Patro et al., 2006).

Although, at present, we are not able to establish the mechanisms underlying the actions of Hcy on the cytoskeleton, they are consistent with the hypothesis of complex actions mediated by Ca^{2+}. Moreover ROS, especially hydroxyl radicals, can produce functional alterations in lipids, proteins and nucleic acids. Oxidative lipid damage, termed lipid peroxidation, produces a progressive loss of membrane fluidity, reduces membrane potential and increases permeability to ions such as Ca^{2+} (Casetta et al., 2005; Naoi et al., 1998). Astrocytic Ca^{2+} responses can be evoked by the release of a number of different transmitters from neurons, activating glial mGluR (Fellin et al., 2004; Porter and McCarthy, 1996). In addition, a growing body of evidence suggests that Ca2+ channels, which control Ca2+ efflux from the endoplasmic reticulum in response to different biochemical signals, are also sensitive to small changes in ROS concentrations or changes in the intracellular thiol

redox status, suggesting that these $^{Ca2+}$ channels serve as physiological redox sensors (Camello-Almaraz et al., 2006).

In addition to activation of mGluRs, NMDA-mediated Ca^{2+} influx contribute to the increased intracellular Ca^{2+} levels contributing to Hcy excitotoxicity. These events could activate the generation of amino acids and free radicals (Dumuis et al., 1988), which can in turn increase the extracellular glutamate concentration by activating NMDA receptors and/or inhibiting glial glutamate uptake (Williams et al., 1989). In this context, our results represent a further evidence of the excitotoxic effect of Hcy targeting the cytoskeleton through NMDA and metabotropic receptors.

Among the intracellular responses to Ca^{2+}, activation of calcineurin, a Ca^{2+}-dependent protein phosphatase (Wang et al., 2005), could be considered, since we have previously demonstrated that calcineurin could be associated with the cytoskeleton, mediating dephosphorylation of glial and neuronal IF proteins in cerebral cortex of rats in response to different insults (de Almeida et al., 2003; Funchal et al., 2005b).

In conclusion, we observed that high Hcy levels target the cytoskeleton of C6 glioma cells and the predominant cytoskeletal response is massive formation of actin- containing filopodia at the cell surface that could be related with Cdc42 activation and increased vinculin. Hcy neurotoxicity involved glutamatergic and oxidative events however we can not exclude more intrincate mechanisms of action. Taking together our results, we could speculate that Hcy at different concentrations elicit complex signaling pathways leading to increased intracellular Ca^{2+} concentrations and a cell response to the injury. Finally, it is feasible that our results may contribute to the understanding of the neural damage provoked by elevated Hcy levels in human brain.

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LEGENDS

Fig. 1. IF phosphorylation in C6-glioma cells. (A) Polyacrylamide gel electrophoresis (SDS–PAGE), autoradiograph and immunoblotting of the cytoskeletal fraction from C6-glioma cells. Lane a, SDS–PAGE of molecular weight standards (kDa) and cytoskeletal fraction from C6-glioma cells stained with Coomassie blue. Lane b, autoradiograph of the Triton-insoluble IF enriched cytoskeletal fraction incubated with 32P orthophosphate. Lanes c and d, immunoblotting of vimentin (Vim) and GFAP, respectively, in the cytoskeletal fraction from C6-glioma cells. (B) Effect of homocysteine (Hcy) on the in vitro phosphorylation of GFAP and Vim in C6-glioma cells. Cell cultures were preincubated with 100 or 500 μM Hcy for 0 or 3 h and incubated with 100 or 500 μM Hcy in the presence of ^{32}P orthophosphate for 1 h. The high-salt Triton insoluble cytoskeletal fraction was extracted and the radioactivity incorporated into GFAP and Vim was measured as described in Material and Methods. (C) Representative immunoblotting of Vim and GFAP in the cytoskeletal fraction. Data are reported as means ± SEM of five diferent experiments expressed as percent of controls. Statistically significant differences from controls as determined by ANOVA followed by Tukey test are indicated: *P <0.01. Representative autoradiographs of C6-glioma cells IF phosphorylation are shown.

Fig. 2. Effects of folate (FA), ascorbic acid (AA) and trolox (Tro) on Hcy-hypophosphorylation in C6-glioma cells. Cells were cultured to confluence in DMEM/F12 10% fetal bovine serum (FBS). The medium was then changed to DMEM/F12 0% FBS and the cells were pre-incubated with FA 5 μM, AA 1 mM and Tro 80 μM 1 h before treatment with 100 or 500 μM Hcy for 3 h. After, C6-glioma cells were incubated in the presence of drugs and ^{32}P orthophosphate for 1 h. The high-salt Triton insoluble cytoskeletal fraction was

extracted and the radioactivity incorporated into GFAP and Vim was measured. Data are reported as means ± SEM of three diferent experiments expressed as percent of controls. Statistically significant differences from controls as determined by ANOVA followed by Tukey test are indicated: *P <0.01. Representative autoradiographs of C6-glioma cells IF phosphorylation are shown.

Fig. 3. Effects of glutamate antagonists on Hcy-hypophosphorylation in C6-glioma cells. Cells were cultured to confluence in DMEM/F12 10% fetal bovine serum (FBS). The medium was then changed to DMEM/F12 0% FBS and the cells were pre-incubated with 50 μM DL-AP5, MCPG or CNQX for 1 h before addition of 100 or 500 μM Hcy for 3 h and in vitro phosphorylation was proceeded. Data are reported as means ± SEM of three diferent experiments expressed as percent of controls. Statistically significant differences from controls as determined by ANOVA followed by Tukey test are indicated: *P <0.01 compared with control group; #P < 0.05 compared with Hcy group. Representative autoradiographs of C6-glioma cells IF phosphorylation are shown.

Fig. 4. Effect of Hcy on microtubule, GFAP and actin organization in C6-glioma cells. Cells were cultured to semi-confluence in DMEM/F12 10% fetal bovine serum (FBS). The medium was then changed to DMEM/F12 0% FBS in the presence or absence of Hcy 100 and 500 μM for 4h. Representative images of Hcy treated cells immunostained with DAPI and monoclonal anti-α-tubulin (A), polyclonal anti-GFAP (C) or phalloidin-fluorescein (E). Scale bar = 20 μm. Western blot analysis of α-tubulin (B), GFAP (D) and β-actin (F) immunocontent in cell homogenate. All lanes received equivalent amount (50μg) of total protein from cell extract. Immunoblot was carried out with monoclonal α-tubulin, anti-GFAP (clone G-A-5), and monoclonal anti-β-actin antibodies diluted 1:500. The blots were

developed using an ECL kit. Data are reported as means ± SEM of four different experiments expressed as percent of controls. Data were statistically analyzed by one-way ANOVA. Representative immunological reactions are shown.

Fig. 5. Effects of Hcy on vinculin immunocontent and cdc42 activation in C6-glioma cells. (A) Western blot analysis of vinculin immunocontent in cell homogenate treated with 100 and 500 μM Hcy for 4 hours. All lanes received equivalent amount (50μg) of total protein from cell extract. Immunoblot was carried out with anti-vinculin antibody diluted 1:500. The blots were developed using an ECL kit. Data are reported as means ± SEM of four diferent experiments expressed as percent of controls. Statistically significant differences from controls, as determined by ANOVA followed by Tukey test are indicated: *P <0.05. Representative immunological reactions are shown. (B) Cdc42 activation in C6-glioma cells treated for 5, 15, 30 minutes and 4 hours with 100 and 500 μM Hcy. Cdc42 activation was mensured using assay Biochem Kit (BK034) from Cytoskeleton (Denver, USA). The blots were developed using an ECL kit. Data are reported as means ± SEM of three diferent experiments expressed as percent of controls. Statistically significant differences from controls, as determined by ANOVA followed by Tukey test are indicated: *P <0.05, **P<0.01. Representative immunological reactions are shown.

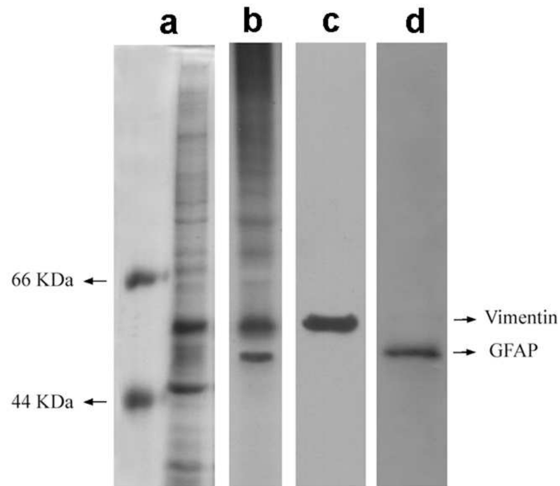
Fig. 6. Effect of folate (FA), trolox (Tro) and ascorbic acid (AA) on Hcy-induced actin organization in C6-glioma cells. Cells were cultured to semi-confluence in DMEM/F12 10% fetal bovine serum (FBS). The medium was then changed to DMEM/F12 0% FBS and cells were pre-incubated with 5 μM FA, 80 μM tro and 1 mM AA 1 h before the 100 and 500 μM Hcy treatment for 4h. Representative images of treated cells immunostained with DAPI and phalloidin-fluorescein. Scale bar = 20 μm.

Fig. 7. Effect of Hcy on the induction of reactive oxygen species (ROS) in C6-glioma cells. Cells were cultured to confluence in DMEM/F12 10% fetal bovine serum (FBS). The medium was then changed to DMEM/F12 0% FBS in the presence or absence of Hcy (100 μM) for 5, 15, 30 minutes, 1 and 4 hours. The intracellular ROS levels were measured with DCF-DA assay. The fluorescence was measured in a fluorescence microplate reader (excitation 485 nm and emission 520 nm). Results are expressed as percent of controls. Statistically significant differences from controls, by one-way ANOVA followed by Tukey's multiple variation test, are indicated: $*P < 0.01$.

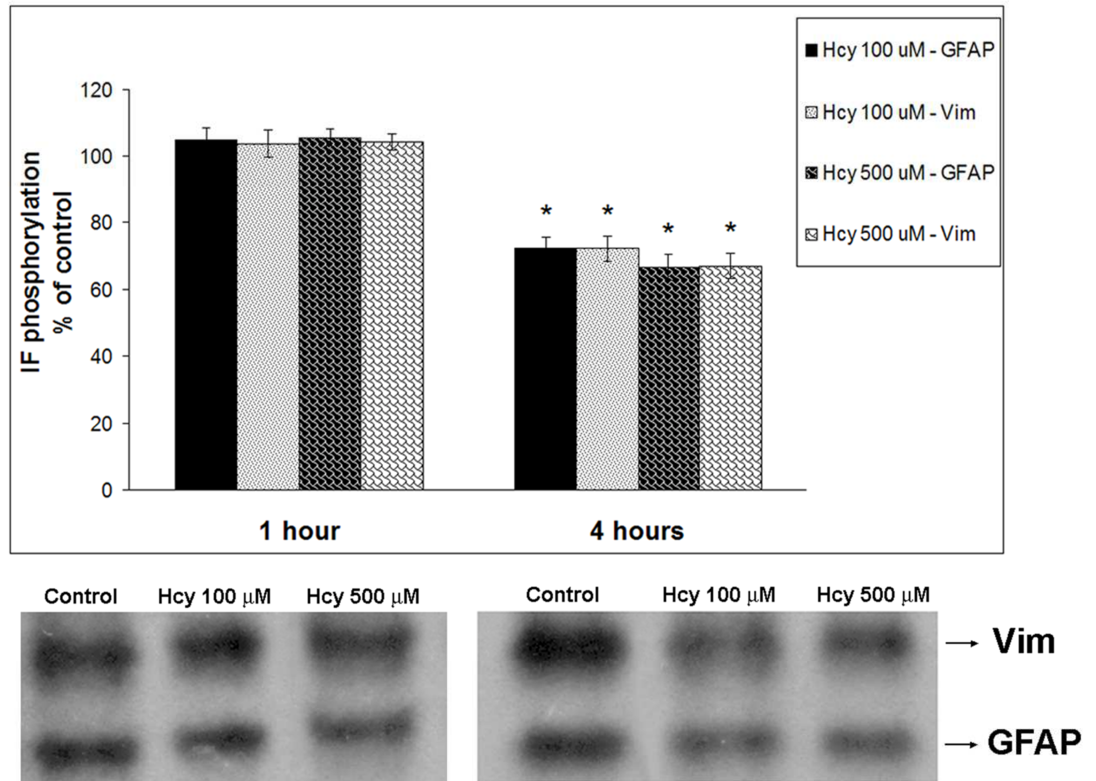
Fig. 8. Effect of glutamate antagonists on Hcy-induced actin reorganization in C6-glioma cells. Cells were cultured to semi-confluence in DMEM/F12 10% fetal bovine serum (FBS). The medium was then changed to DMEM/F12 0% FBS and cells were pre-incubated with 10 μM DL-AP5, 50μM MCPG and 25μM CNQX for 1 h before addition of 100 and 500 μM Hcy for 4h. Representative images of treated cells immunostained with DAPI and phalloidin-fluorescein. Scale bar = 20 μm.

FIGURE 1

A



B



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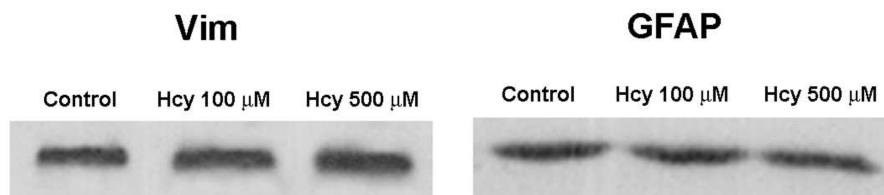


FIGURE 2

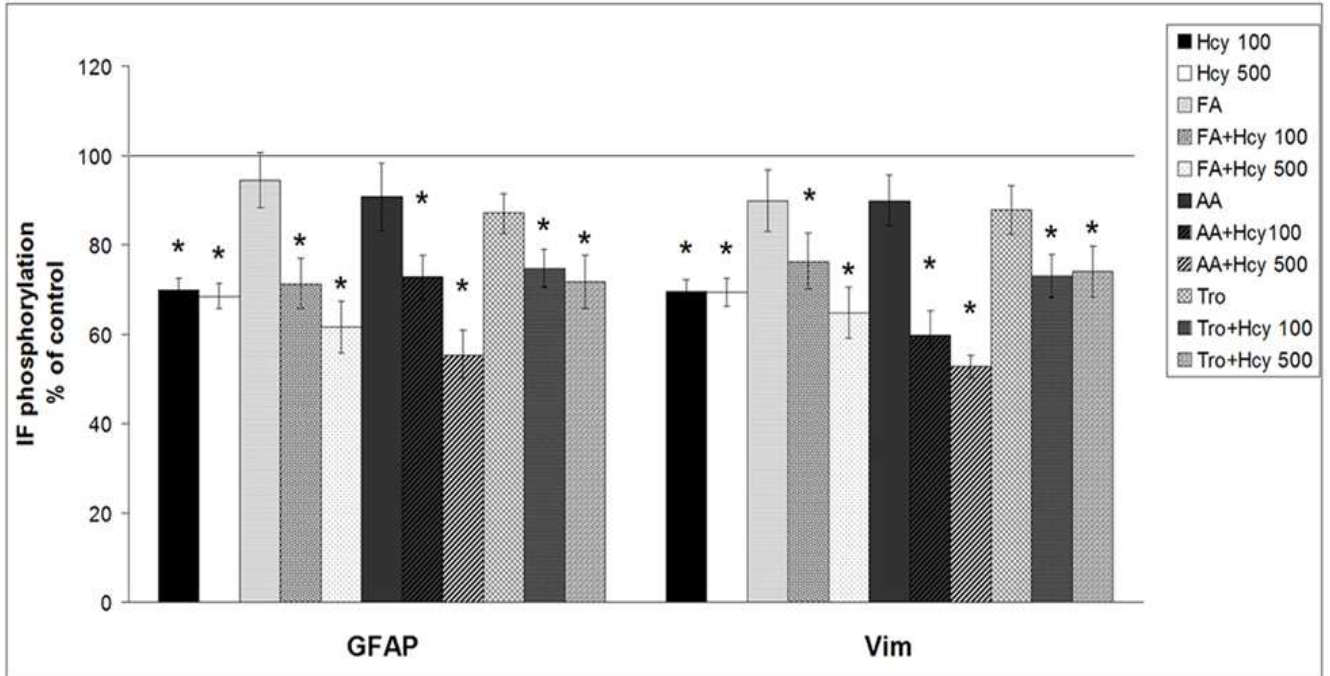


FIGURE 3

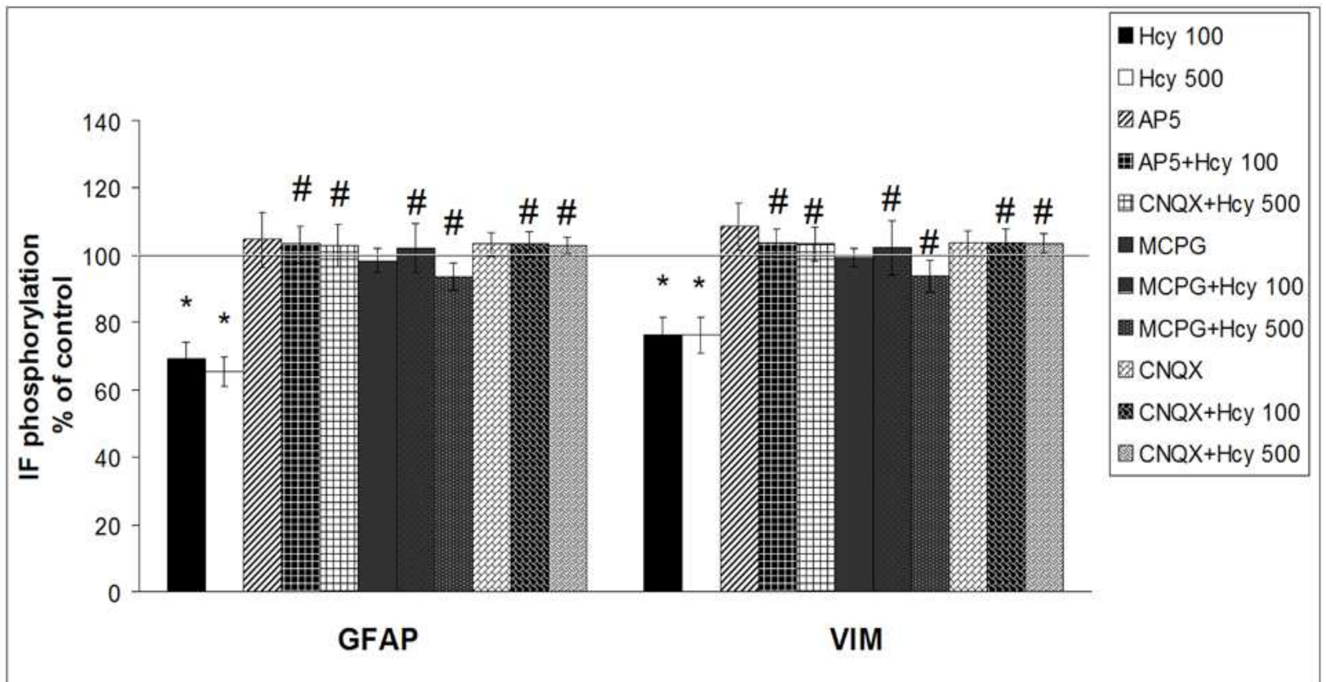


FIGURE 4

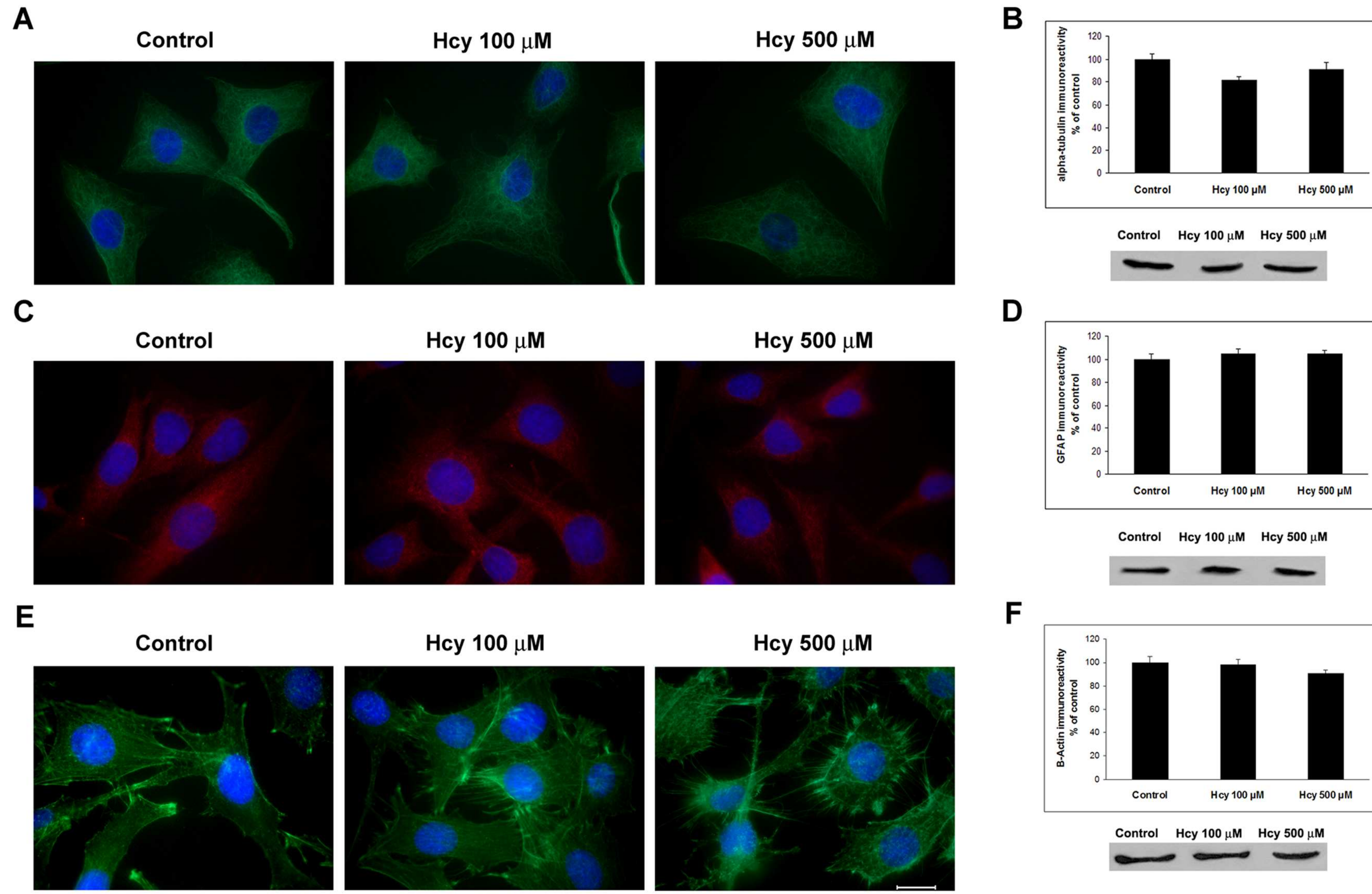
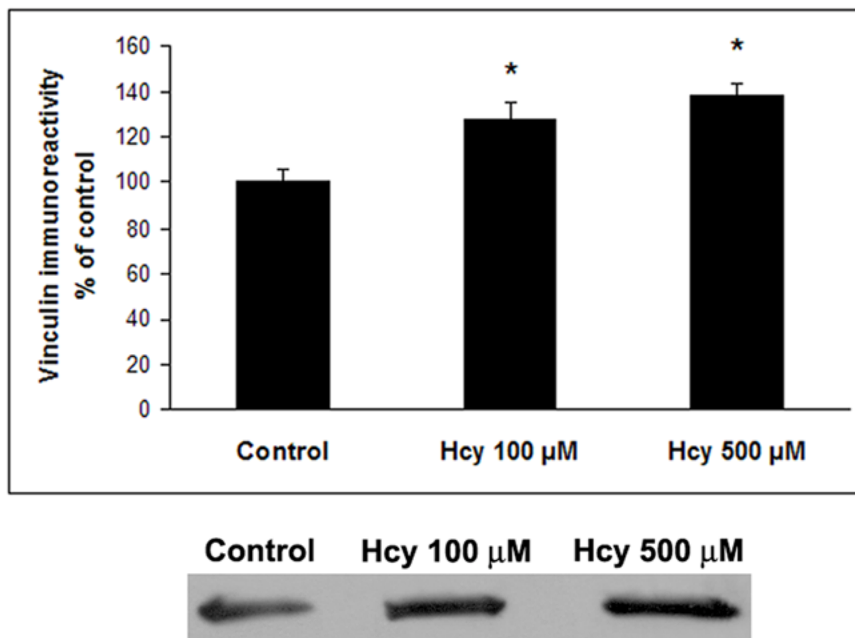


FIGURE 5

A



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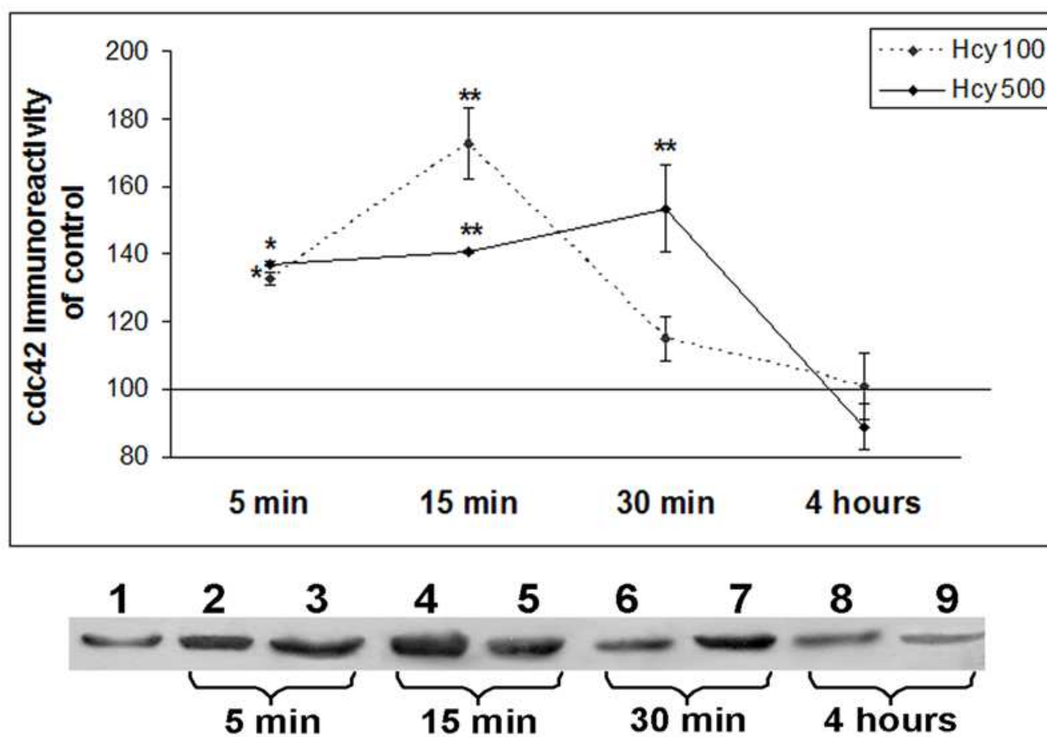


FIGURE 6

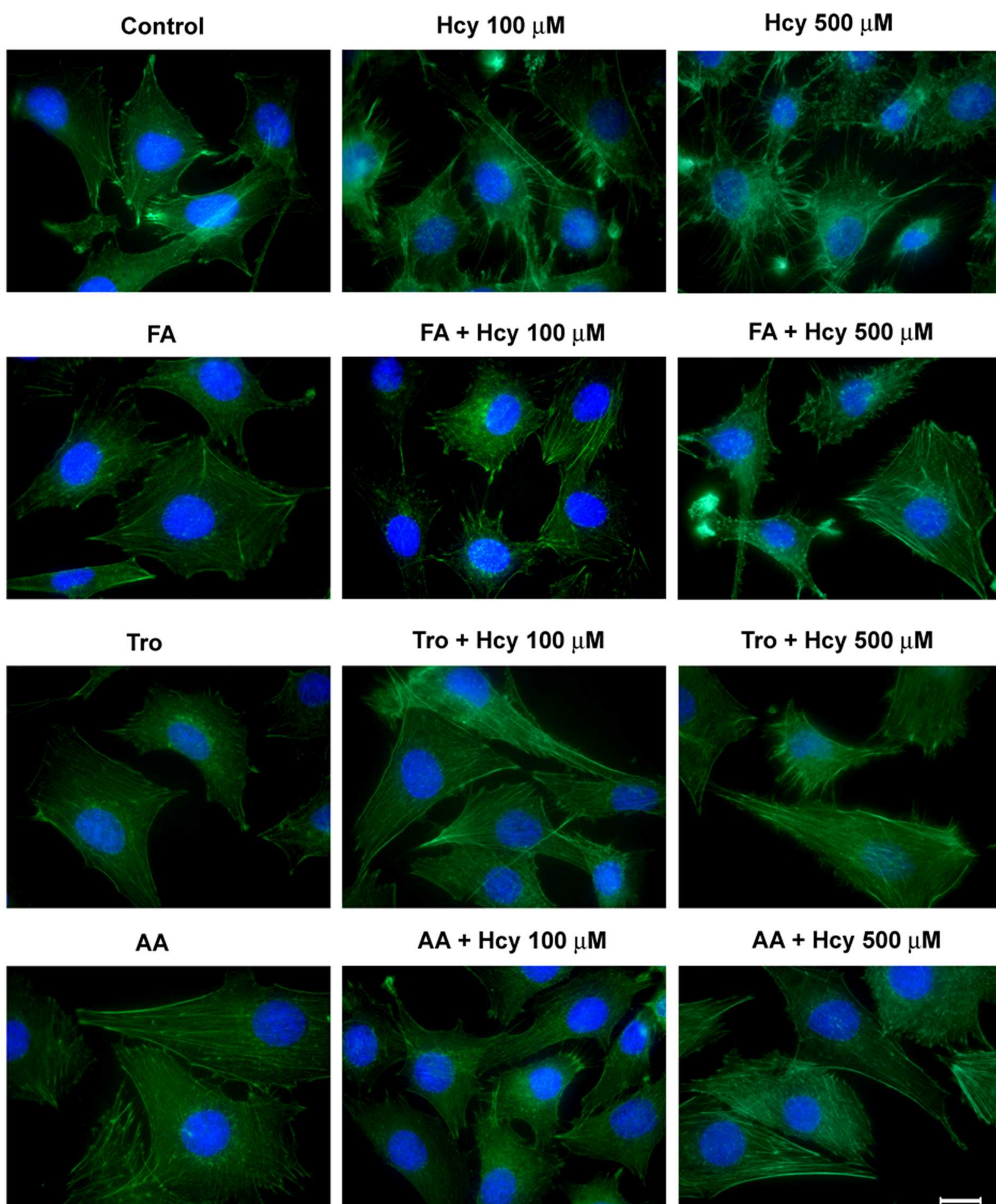


FIGURE 7

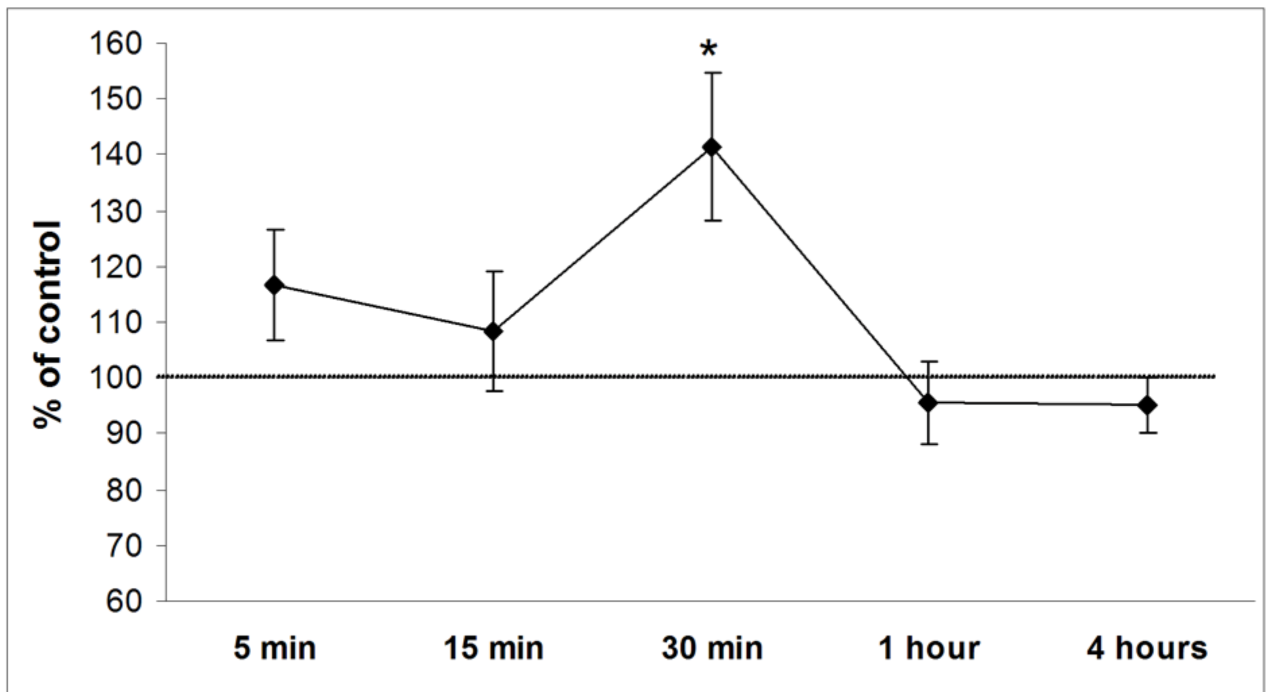
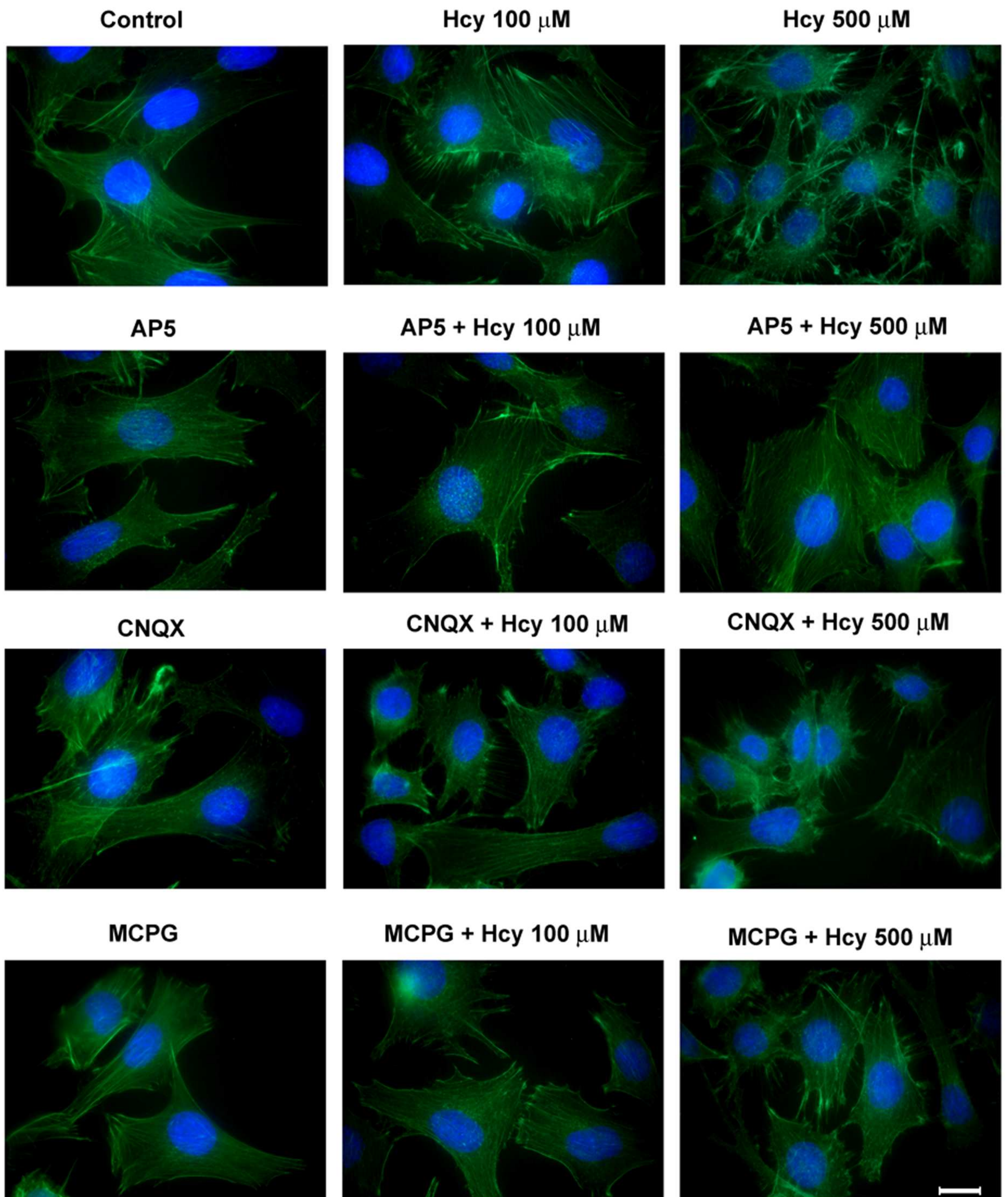


FIGURE 8



PARTE III

1. DISCUSSÃO

As doenças neurodegenerativas estão entre os desafios mais enigmáticos e problemáticos da medicina (Haass e Selkoe, 2007). Como as pesquisas dessas doenças passaram de fenômenos descritivos para análises mecanísticas, tornou-se cada vez mais evidente que os processos básicos envolvidos são de natureza multifatoriais, causados por fatores genéticos, ambientais e endógenos (Cavalli et al., 2008). Alterações na dinâmica dos constituintes do citoesqueleto celular são muito relevantes para um melhor entendimento de algumas doenças neurodegenerativas, tendo em vista que o mesmo desempenha um papel fundamental na manutenção da forma assimétrica, estrutura polarizada e da plasticidade dos neurônios, fatores que são essenciais para a fisiologia neuronal. Devido a sua participação na neuritogênese, a reorganização do citoesqueleto pode ocasionar comprometimento na neurotransmissão. Na doença de Alzheimer, placas amilóides e emaranhados neurofibrilares, os quais são formados por filamentos quase inteiramente constituídos pela proteína tau associada aos microtúbulos, são as duas maiores alterações neuropatológicas presentes no cérebro. Outro exemplo de anormalidades do citoesqueleto presente em doenças neurodegenerativas são os corpos de Lewy que são considerados um marco citoplasmático da doença de Parkinson (Benitez-King et al., 2004; Feng, 2006; Gong e Iqbal, 2008).

Estudos epidemiológicos e experimentais relacionam HHCY com condições neurodegenerativas incluindo doença de Alzheimer, acidente vascular cerebral e doença de Parkinson (Mattson e Shea, 2003; Zou e Banerjee, 2005). Entretanto, os mecanismos que medeiam a patogênese relacionada com Hcy não são totalmente esclarecidos. Modelos experimentais têm identificado importantes fenômenos relacionados a neurotoxicidade induzida por altas concentrações de Hcy, como apoptose de células neurais (Blaise et al., 2007; Kruman et al., 2000; Kruman et al., 2004), estimulação de receptores NMDA, aumento

dos níveis de Ca^{+2} citosólico e formação de espécies reativas de oxigênio (ROS) (Ho et al., 2002; Lipton et al., 1997; Tjiattas et al., 2004). Além disso, existem poucas evidências que relacionam efeitos neurotóxicos da Hcy e citoesqueleto celular, no entanto, podemos citar a indução de fosforilação da tau via PP2A (Luo et al., 2007) e os efeitos da Hcy na neurulação cranial em embriões de pinto através de mecanismos que envolvem a dinâmica da actina (Brouns et al., 2005). Considerando o exposto acima, neste trabalho utilizamos modelos experimentais de homocistinúria com o objetivo de avaliar os efeitos da Hcy sobre o citoesqueleto de células neurais de ratos.

1.1 Estudo *in vivo* dos efeitos da Hcy

Primeiramente, utilizamos um modelo experimental de HHCY desenvolvido por Streck et al (2002), no qual os animais apresentam níveis plasmáticos de Hcy similares aos dos pacientes homocistinúricos. Esse modelo tem contribuído para um melhor entendimento dos danos neurais provocados pela Hcy, demonstrando alterações em diversos parâmetros bioquímicos, tais como: deficiência na memória (Streck et al., 2004), inibição da atividade da $Na^{+}/K(^{+})$-ATPase (Matte et al., 2004; Matte et al., 2007; Streck et al., 2002), redução da atividade da butiril-colinesterase (Stefanello et al., 2005), aumento no dano tecidual em hipocampo causado pela deprivação de oxigênio e glicose (Tagliari et al., 2006) e o aumento do dano ao DNA (Matte et al., 2008).

No presente trabalho verificamos que o córtex cerebral e o hipocampo dos ratos jovens cronicamente tratados com Hcy apresentavam uma expressão alterada de subunidades dos FIs. Esse mecanismo de alteração genômica desencadeado pela Hcy está de acordo com estudos prévios que apontam ativação de fatores de transcrição em resposta ao aumento dos níveis de Hcy (Au-Yeung et al., 2004; Lee et al., 2005). Além disso, é bem estabelecido que a

Hcy induz dano ao DNA devido a perturbações no ciclo de metilação do mesmo (Wainfan e Poirier, 1992). Outro aspecto relevante é a seletividade dessas alterações, tendo em vista que no córtex ocorreu uma diminuição na expressão de GFAP (FI astrocitário) e no hipocampo observou-se um aumento na expressão de NF-H e NF-L (FIs neuronais). As razões dessa seletividade podem estar relacionadas com diferenças na suscetibilidade dos FIs corticais e hipocampais ao tratamento crônico com Hcy, bem como as interações astrócito-neurônio, uma vez que a modulação da função astrocitária pode ter efeitos nocivos na função neuronal (Enokido, 2007; Seifert et al., 2006).

Verificou-se também que o hipocampo é a estrutura mais vulnerável aos efeitos deletérios desencadeados pela Hcy nos FI do citoesqueleto neural. A vulnerabilidade do cérebro em desenvolvimento é dependente de duas principais questões: o primeiro fator se refere a capacidade do agente ou seu metabólito ativo em atingir o sistema nervoso e o segundo fator se relaciona ao período de exposição ao agente. Quando a exposição do agente tóxico coincide com a ontogenia dos processos de desenvolvimento, ocorre uma maior suscetibilidade de efeitos adversos, podendo interferir com os eventos moleculares do desenvolvimento. Em geral, se a exposição ocorrer antes ou depois do desenvolvimento de um órgão, este será menos vulnerável a perturbação do que se o insulto ocorrer durante o seu desenvolvimento (Rice e Barone, 2000). O hipocampo e o cerebelo são as únicas estruturas cerebrais em que normalmente ocorre neurogênese pós-natal. No córtex cerebral de ratos, a neurogênese ocorre totalmente no período pré-natal, do 13° ao 22° dia gestacional, enquanto nas células granulares do giro denteado do hipocampo, a neurogênese é observada a partir do 19° dia de gestação até o 19° dia de vida pós-natal (Bayer et al., 1993; Rice e Barone, 2000). Considerando que o tratamento crônico com Hcy abrange uma boa parte desse período de vulnerabilidade do hipocampo e que o citoesqueleto possui um importante papel na neurogênese (Jalava et al., 2007; Kim et al., 2002; Lopez-Picon et al., 2004; Rami, 2003),

podemos inferir que o hipocampo foi mais suscetível a toxicidade da Hcy sobre o citoesqueleto neural devido a perturbações na neurogênese desta estrutura. Essa hipótese está de acordo com Rabaneda e colaboradores (2008) que descreveram efeitos da Hcy na neurogênese do giro denteado do hipocampo. Outros grupos também descreveram o comprometimento na neurogênese do giro denteado do hipocampo de camundongos submetidos a uma dieta com baixos níveis e folato e conseqüentemente altos níveis de Hcy (Kronenberg et al., 2008; Kruman et al., 2005).

Tendo em vista que a fosforilação é um importante mecanismo de regulação da estequiometria dos NFs (Ackerley et al., 2003; Garcia et al., 2003; Rao et al., 2003; Sanchez et al., 2000; Shea e Chan, 2008; Veeranna et al., 2008), o fato de que o tratamento com Hcy não alterou a fosforilação dos FIs no córtex e principalmente no hipocampo dos animais tratados, inicialmente nos surpreendeu. No entanto, esses resultados poderiam ser explicados com base na dessensibilização de receptores de membrana em resposta a uma exposição prolongada a um estímulo, com subseqüente redução de sua resposta celular (Alberts, 2008). Durante o tratamento crônico, com constante estímulo de Hcy, receptores, como os glutamatérgicos, poderiam ter se tornado insensíveis ao estímulo, consequentemente as atividades enzimáticas moduladas pelo sistema glutamatérgico, como por exemplo, o sistema fosforilante associado ao citoesqueleto, teriam retornado a uma situação de insensibilidade. Sauls e colaboradores (2004) descreveram uma adaptação ao tratamento crônico com Hcy em coelhos jovens, evidenciada pela redução da resposta de aumento do estresse oxidativo induzido pela Hcy.

Ao final do tratamento crônico com Hcy, observamos alteração na expressão e na estequiometria dos FIs. Sabe-se que a expressão gênica é regulada por mecanismos complexos que incluem vias de sinalização tais como a via da MAPK (Marek et al., 2004; Nishina et al., 2008; Obata et al., 2003; Romao et al., 2008). Embora a MAPK não esteja

ativada ao final do tratamento, podemos supor que esta via tenha sido ativada ou inibida em algum período durante o desenvolvimento cerebral, levando as alterações de expressão observadas nas proteínas estudadas. Com base na análise estequiométrica dos FIs astrocitários dos ratos tratados, podemos sugerir uma redução no "turnover" de GFAP devido, provavelmente, a falha no sistema ubiquitina-proteosoma encarregado de destruir proteínas anormais (Bachetti et al., 2008a; Mignot et al., 2007). No caso do hipocampo, nós propomos que esse "turnover" reduzido poderia levar ao acúmulo de GFAP. O acúmulo de FI astrocitário levando a formação de agregados é encontrado em doenças neurodegenerativas, como a doença de Alexander (Bachetti et al., 2008b).

Além disso, a estequiometria dos NFs de hipocampo no modelo de HHCY é consistente com uma diminuição na quantidade de filamentos enriquecidos em projeções laterias, bem como o favorecimento de subunidades dos NF solúveis. Esses achados podem contribuir para a formação de inclusões tóxicas de FI, consideradas marcos patológicos para algumas doenças neurodegenerativas (Beaulieu e Julien, 2003; He e Hays, 2004; Julien e Beaulieu, 2000; Xiao et al., 2006).

Essa incongruência entre alterações estequiométricas e insensibilidade do sistema fosforilante no modelo experimental de HHCY também pode ser uma conseqüência da complexidade das vias de sinalização desencadeadas pela Hcy durante o desenvolvimento cerebral, tendo em vista que a fosforilação dos FIs pode ser um alvo e, ao mesmo tempo, um modulador de múltiplas cascatas de sinalização celular (Omary et al., 2006).

Para compreendermos melhor os mecanismos moleculares desencadeados pela Hcy sobre a fosforilação dos FIs neurais durante o desenvolvimento do sistema nervoso central, desenvolvemos uma abordagem *in vitro*, utilizando fatias de córtex cerebral e hipocampo de ratos jovens.

1.2 Estudo *in vitro* dos efeitos da Hcy

Nos estudos *in vitro*, confirmamos a maior vulnerabilidade do hipocampo nas ações da Hcy sobre o citoesqueleto neural. Observamos também três peculiaridades nos efeitos da Hcy sobre a fosforilação dos FIs em fatias de córtex cerebral e hipocampo de ratos: a primeira se refere ao fato de que os efeitos foram observados apenas em hipocampo; a segunda, diz respeito ao fato de que apenas o citoesqueleto dos animais de 17 dias de idade foi sensível a injúria; e a terceira refere-se ao efeito antagônico das duas concentrações estudadas sobre o citoesqueleto neural (Hcy 100 e 500 μM).

Com relação ao efeito seletivo da Hcy em fatias hipocampais, essa observação é consistente com os nossos presentes resultados *in vivo* e com evidências prévias de excitotoxicidade induzida pela Hcy em hipocampo de ratos (Algaidi et al., 2006; Baydas et al., 2006; Baydas et al., 2005; Kim et al., 2007; Kruman et al., 2000; Lazarewicz et al., 2003; Robert et al., 2005; Robert et al., 2004). Como exemplo, Fukui e colaboradores (2002) e Streck e colaboradores (2004) relataram deficiências comportamentais associadas a Hcy e Baydas e colaboradores (2005) descreveram distúrbios no aprendizado e na memória envolvendo mecanismos de geração de ROS e alterações na plasticidade sináptica em hipocampo de ratos

Com respeito à suscetibilidade do hipocampo de ratos de 17 dias de idade aos efeitos da Hcy, é importante salientar que ratos de 17-19 dias pós-natal têm sido utilizados como modelos experimentais de injúrias cerebrais traumáticas leves a moderadas ocorridas em crianças, enfatizando 17 dias como uma idade crítica do desenvolvimento desta estrutura cerebral em ratos. (Gurkoff et al., 2006; Huh et al., 2008; Prins e Hovda, 1998; Yager e Thornhill, 1997). Por outro lado, é difícil estabelecer as bases moleculares responsáveis pela janela de vulnerabilidade aos insultos, observada nos animais de 17 dias de idade,

desencadeados por diferentes concentrações de Hcy. Entretanto, podemos propor que esses achados possam estar relacionados com a intensa neurogênese, a qual aumenta a vulnerabilidade hipocampal durante esta fase do desenvolvimento.

A hiper e a hipofosforilação, das subunidades de FIs induzidas pela Hcy 100 μM e 500 μM, respectivamente, em hipocampo de ratos de 17 dias de idade são efeitos antagônicos muito interessantes. Efeitos opostos da Hcy já tinham sido relatados em outros parâmetros bioquímicos (Lee et al., 2002; Luchowska et al., 2005; Panganamala et al., 1986; Stazka et al., 2005; Zhang et al., 2008a). Essas observações salientam ainda mais a assombrosa complexidade dos sistemas de sinalização celular em resposta a determinados insultos, envolvendo múltiplas e interconectadas cadeias de transmissão.

Nesse contexto, podemos propor mecanismos baseados na ativação de diferentes vias de sinalização mediadas pelo Ca^{2+}, a semelhança do que ocorre nos dois tipos de plasticidade sináptica hipocampal (LTP e LTD) (Bear, 2008). Com base nesta proposta, o estímulo da Hcy levaria a entrada de Ca^{+2} na região pós-sinaptica, originando ondas de Ca^{+2} intracelulares com freqüências de oscilações que refletiriam a força do estímulo e levariam a uma resposta celular específica.

A hiperfosforilação dos FI do citoesqueleto neural induzida por 100 μM de Hcy (concentração relacionada a HHCY moderada) em hipocampo de ratos de 17 dias de idade foi mediada por mecanismos envolvendo a mobilização de Ca^{+2} através de receptores NMDA, canais de Ca^{+2} dependentes de voltagem tipo L e receptores de rianodina presentes no retículo endoplasmático, ou seja, modificações no influxo e concentrações intracelulares de Ca^{+2}. No entanto, a hipofosforilaçao induzida por 500 μM de Hcy (concentração relacionada a HHCY grave) na mesma idade e estrutura cerebral, foi mediada pelos mesmos mecanismos de sinalização envolvendo a mobilização de Ca^{+2} desencadeados pela 100 μM, com exceção do envolvimento dos canais de Ca^{+2} dependentes de voltagem (VDCC) do tipo L.

Conseqüentemente, esses mecanismos promovidos pelas ações da Hcy sobre a homeostase do Ca^{+2} culminam na ativação de diferentes enzimas dependentes de Ca^{+2}: quinases no caso da Hcy 100 μM (PKCam II e PKC) e fosfatases no caso da 500 μM (PP2B). Esses achados nos possibilitam inferir que a opção da célula em ativar quinases ou fosfatases, em resposta a diferentes concentrações de Hcy, é dependente da amplitude e da freqüência de duração das oscilações de Ca^{+2}. Nesse contexto, evidências prévias mostraram que a toxicidade da Hcy no SNC é relacionada com o incremento nos níveis de Ca^{+2} intracelular (Ho et al., 2003; Ho et al., 2001; Ho et al., 2002; Kim et al., 2007; Lipton et al., 1997; Obeid e Herrmann, 2006; Tjiattas et al., 2004; Zieminska e Lazarewicz, 2006; Zieminska et al., 2003; Zimny, 2008).

Os VDCC do tipo L são ativamente envolvidos no controle da excitabilidade da membrana, plasticidade sináptica e expressão gênica, entre outros processos (Gallin e Greenberg, 1995; Lipscombe et al., 2002; Surmeier, 2007; Thibault et al., 2007). Por um lado, os VDCC aumentam os níveis de Ca^{2+} intracelular, ativando a PP2B, por outro lado, a PP2B é um candidato potencial na modulação da atividade de VDCC do tipo L (Norris et al., 2008). Norris e colaboradores (2005) demonstraram que a ativação da PP2B, em cultura de neurônios hipocampais é capaz de permitir, bem como amplificar a atividade dos VDCCs do tipo L. Tendo em vista que o efeito da Hcy 500 μM foi mediado pela PP2B, entre outras fosfatases, podemos supor que os VDCCs do tipo L podem estar envolvidos apenas indiretamente com tais efeitos. O fato de que o bloqueador de VDCC tipo L, verapamil, não ter prevenido a hipofosforilaçao induzida pela Hcy 500 μM na mesma concentração em que previniu a hiperfosforilação induzida pela Hcy 100 μM, pode ser uma evidência de que na concentração de 500 μM, a Hcy não estaria ativando diretamente estes canais, contudo esta possibilidade precisa ser comprovada experimentalmente.

Além das quinases e fosfatases dependentes de Ca^{+2}, a hiperfosforilação induzida pela Hcy 100 μm foi mediada por MAPK, PI3K e fosfolipase C (PLC), enquanto a

hipofosforilação desencadeada pela Hcy 500 μm foi uma decorrência da ativação de vias de sinalização envolvendo PP21, PP2A, PP2B, mGluR e iGluR não-NMDA.

O "cross-talk" entre as múltiplas vias de sinalização que a Hcy foi capaz de ativar para gerar seus dois efeitos antagônicos sobre o sistema fosforilante associado ao citoesqueleto neural é extremamente amplo. Tendo em vista que a Hcy ou seus metabólitos podem atuar ativamente em receptores NMDA (Do et al., 1988; Kim et al., 2007; Lazarewicz et al., 2003; Ramos Coutinho et al., 2008; Robert et al., 2005; Zieminska e Lazarewicz, 2006), e considerando apenas esse receptor, observamos que o mesmo pode interagir (modulando ou sendo modulado) com todos os outros receptores ou sinalizadores celulares recrutados nos efeitos da Hcy 100 μM bem como da Hcy 500 μM sobre a fosforilação dos FIs de hipocampo de ratos de 17 dias. A seguir, algumas dessas interações serão discutidas.

A atividade do receptor NMDA pode ser regulada por uma variedade de proteínas fosfatases e quinases como a PP2B (Krupp et al., 2002; Lieberman e Mody, 1994; Raman et al., 1996; Tong e Jahr, 1994; Tong et al., 1995), a PP1 e a PP2A (Wang et al., 1994; Westphal et al., 1999), a PKC (Chen e Huang, 1991,1992; Lan et al., 2001; Lu et al., 1999; Xiong et al., 1998) e a PKA (Cerne et al., 1993; Crump et al., 2001; Raman et al., 1996). Inclusive, interações entre subunidades do receptor NMDA e PP2A podem sugerir um novo mecanismo de sinalização mediado pelos receptores NMDA, proporcionando outras funções para esses receptores, além das tradicionalmente conhecidas (Chan e Sucher, 2001). A via da MAPK também possui uma significativa influência sobre a neuroquímica dos receptores NMDA, regulando suas propriedades fisiológicas, bioquímicas e biofísicas, e seu potencial papel na fisiopatologia de algumas doenças (Haddad, 2005).

Além disso, a regulação da expressão de receptores NMDA na membrana celular e da densidade sináptica é critico para a homeostase e plasticidade sináptica (Bear e Abraham, 1996; Carroll e Zukin, 2002; Turrigiano e Nelson, 2000; Wenthold et al., 2003). Qualquer

disfunção nos mecanismos celulares que controlam esses processos pode resultar em desordens como doenças neurodegenerativas (Cull-Candy et al., 2001). Muitos estudos apontam que a regulação do tráfego dos receptores NMDA do retículo endoplasmático para a superfície celular, local onde os mesmos desencadeiam suas ações, pode ser via fosforilação (Scott et al., 2001; Xia et al., 2001) e que a PKA (Crump et al., 2001) e a PKC (Lan et al., 2001) podem estar envolvidas nessa modulação (Scott et al., 2003; Scott et al., 2001).

O receptor NMDA pode servir como uma âncora para a translocação da PKCam II posicionando-a próximo a entrada de Ca^{+2} e aproximando-a de seus substratos. Mais interessante ainda é o fato que os receptores NMDA se ligam a quinase no seu estado ativo e a mantém ativa mesmo quando dissociada da calmodulina (Bayer e Schulman, 2001).

A ativação de mGluR pode aumentar a resposta provocada por receptores NMDA em hipocampo, estriado, córtex e medula espinhal, sugerindo que as interações funcionais entre mGluR e receptores NMDA são de extrema significância (Aniksztejn et al., 1992; Attucci et al., 2001; Doherty et al., 1997; Harvey e Collingridge, 1993; Kotecha et al., 2003; Mannaioni et al., 2001; Pisani et al., 1997; Ugolini et al., 1997).

Como todos esses sistemas podem atuar modulando ativamente o sistema fosforilante associado ao citoesqueleto celular de forma a tornar essa estrutura altamente dinâmica (Eriksson et al., 2004; Frizzo et al., 2004; Funchal et al., 2004; Kobayashi et al., 2001; Kommers et al., 2002; van den Heuvel et al., 2002; Woll et al., 2007; Zamoner et al., 2006; Zamoner et al., 2008b), e com base nessas e muitas outras possíveis inter-relações entre os diversos sistemas de sinalização celular que podem ser desencadeados por um insulto neurodegenerativo, como altas concentrações de Hcy, a compreensão da maneira como a célula faz a opção de uma determinada resposta celular (ser insensível, hiperfosforilar ou hipofosforilar os FI de hipocampo de ratos) ainda é um mistério e um enorme desafio.

1.3 Estudo dos efeitos da Hcy em culturas celulares

O uso de culturas de células neurais nos possibilita a obtenção de dados que, devido à complexidade das interações celulares, seriam difíceis ou impossíveis de se detectar em experimentos usando tecido intacto (Hansson, 1989). Considerando que no modelo *in vivo* de HHCY ocorreu uma diminuição da expressão de GFAP em córtex cerebral dos ratos cronicamente tratados com Hcy, decidimos avaliar os efeitos da Hcy em cultura de células neurais corticais. Primeiramente avaliamos os efeitos da Hcy sobre o citoesqueleto de astrócitos e neurônios corticais e posteriormente ampliamos o estudo utilizando a linhagem celular C6, que apresenta características gliais (Bissell et al., 1974; Kumar et al., 1984; Parker et al., 1980; Pfeiffer et al., 1970; Vernadakis et al., 1991; Vielkind et al., 1990).

Observamos uma insensibilidade dos neurônios corticais aos efeitos da Hcy com relação aos parâmetros estudados. Esse resultado confirma os estudos *in vitro* e *in vivo* anteriormente discutidos, nos quais observamos que as subunidades dos FIs neuronais associadas ao citoesqueleto de córtex cerebral de ratos também não sofreram qualquer alteração no seu sistema fosforilante, imunoconteúdo ou expressão promovida pela Hcy. Além disso, essa ausência de efeito pode ser atribuída a imaturidade da cultura e a quantidade de magnésio (Mg^{+2}) (812 µM) contido no meio de cultura (Neurobasal). A composição do meio e a idade da cultura são dois fatores que influenciam a expressão de receptores glutamatérgicos, bem como as propriedades desses receptores (Balazs et al., 1992; Brewer et al., 1993; Hoffmann et al., 1997; Li et al., 1998; Priestley et al., 1996). Considerando que a Hcy age através de receptores glutamatérgicos (Lazarewicz et al., 2003; Lipton et al., 1997; Robert et al., 2004; Zieminska e Lazarewicz, 2006; Zieminska et al., 2003), podemos sugerir que a cultura de neurônios corticais utilizada ainda não apresentava quantidade e funcionalidade suficiente desses receptores para a Hcy poder desencadear seus efeitos

neurotóxicos (Chung et al., 2005; Ghiani et al., 2006; Kovacs et al., 2001; Lockhart et al., 2007; Nagy et al., 2003). Além disso, se a concentração de Mg^{+2} fosse reduzida, aumentaria a sensibilidade do receptor NMDA, como demonstrado em outros estudos (Arvanian e Mendell, 2001; Dost e Rundfeldt, 2000).

Com respeito a viabilidade celular, demonstramos uma indução de apoptose em astrócitos corticais expostos a 500 µM de Hcy, enquanto as células da linhagem C6 foram resistentes a essa ação tóxica. Esses resultados estão de acordo com a susceptibilidade astrocitária aos insultos de Hcy (Blaise et al., 2007; Jin e Brennan, 2008; Maler et al., 2003; Zhang et al., 2008b) e a propriedade das células de gliomas serem mais resistentes a estímulos apoptóticos (Alleyne et al., 1999; Morrone et al., 2005; Takano et al., 2001).

Alterações do citoesqueleto de actina, em resposta a sinais celulares, podem ocasionar complicações relevantes em diversas funções dos astrócitos, como o transporte, a motilidade celular, o suporte metabólico, a excitotoxicidade e o estresse oxidativo (Aschner, 2000; Danbolt, 2001; Dent e Gertler, 2003; Hazrati et al., 2008; Lamigeon et al., 2001; Nicchia et al., 2008; Sergeeva et al., 2000). Além disso, disfunções astrocitárias e desregulação de suas funções especificas estão associadas com várias doenças neurodegenerativas (Hedlund e Isacson, 2008; Seifert et al., 2006; Ting et al., 2007).

Considerando as alterações na dinâmica do citoesqueleto celular, observamos que a sensibilidade de astrócitos e de células C6 foram diferentes com relação ao tempo de resposta ao insulto e ao tipo de efeito desencadeado, principalmente em relação ao citoesqueleto de actina e GFAP. Astrócitos apresentaram retração no citoplasma com redistribuição dos filamentos de actina e GFAP apenas após 24 horas de tratamento com Hcy 100 µM, enquanto nas células C6 observou-se um grande aumento no número de filipódios e não alteração do citoesqueleto de GFAP após 4 horas de tratamento com Hcy 100 e 500 µM. Essas diferenças podem ser atribuídas às vias de sinalização ativadas em resposta a Hcy, as quais podem ser

desencadeadas de forma distinta em astrócitos e células C6. Por exemplo, as vias de sinalização dependentes de Ca^{+2} e de cAMP podem ser diferentes em astrócitos e nas células C6. A respeito disso, Vatter e colaboradores (2005) descreveram que a entrada de Ca^{+2}, após depleção por ácido ciclopiazônico, é muito maior em culturas de células C6 do que em culturas de astrócitos e que essas culturas podem apresentar padrões diferenciados de expressão para as enzimas fosfodiesterases cAMP-específicas (PDE1 e PDE4), podendo originar diferentes níveis de Ca^{+2} e cAMP intracelulares e conseqüentemente, distintos efeitos biológicos. Outro exemplo é observado com os receptores purinérgicos P2Y1, P2Y2 e P2Y12 que estão presentes na mitocôndria de astrócitos e de células C6, pois há diferenças significativas em relação à distribuição mitocondrial desses receptores. A maior diferença refere-se a co-localizaçao do receptor P2Y1, em que no glioma C6 observou-se uma diminuição de 60-75% em comparação com astrócitos. Essa diferença pode influenciar as vias de transdução de sinais intracelulares desencadeadas por nucleotídeos em astrócitos e células C6, além de interferir no processo de malignidade observado pela linhagem celular (Krzeminski et al., 2007).

A extrema dinâmica dos filamentos de actina pode promover a longevidade celular, enquanto a indução de estabilização desses filamentos está associada a vias de apoptose celular (Boldogh e Pon, 2006; Gourlay e Ayscough, 2005a,b,c; Odaka et al., 2000). Nossos achados são consistentes com esta relação entre o citoesqueleto de actina e rotas de apoptose celular, pois o único efeito de indução de apoptose promovido pela Hcy (Hcy 500 μM em astróctios) foi associado com o não remodelamento do citoesqueleto de actina.

O grande aumento no número de filipódios desencadeado pela Hcy na linhagem C6, pode ser relacionado com a propriedade tumoral dessas células, uma vez que Werbowetski e Shivers (2001) têm relacionado o aumento de filipódios com agregação e migração celular, os quais podem estar envolvidos na formação das metástases. Além disso, os papéis fisiológicos

dos filipódios poderiam permitir interações altamente dinâmicas entre as sinapses e a interface glia-vascular, importantes para a propagação do sinal intracelular (Benediktsson et al., 2005; Hirrlinger et al., 2004; Mulligan e MacVicar, 2004; Simard et al., 2003). Consistente com a formação de filipódios, a atividade da GTPase monomérica, cdc42, foi aumentada após o tratamento com Hcy. Essa ativação de cdc42 pode levar a formação de contatos focais contendo vinculina na periferia e ao longo das pontas do filipódio (Nobes e Hall, 1995), resultados que evidenciamos com o aumento do imunoconteúdo de vinculina observado nas células de glioma tratadas. Em contrapartida, esse aumento de vinculina pode ser requerido para a estabilização do filipódio (Varnum-Finney e Reichardt, 1994). Enfatizando essa hipótese, verificamos que astrócitos corticais tratados com Hcy não apresentaram alteração no imunoconteúdo de vinculina, fato que pode ser devido ao tipo de alteração no citoesqueleto de actina, ou seja, em astrócitos não observamos aumento de filipódios e sim, retração do citoplasma com reorganização de actina.

Mecanismos envolvendo estresse oxidativo mediaram efeitos promovidos pela Hcy na reorganização do citoesqueleto em astrócitos e células C6. Esses resultados corroboram com inúmeras evidências na literatura que descrevem uma intima relação entre os efeitos da Hcy e estresse oxidativo em diversos tecidos (Ansari e Bhandari, 2008; Garcia et al., 2008; Kassab et al., 2008; Zhu et al., 2008; Zou e Banerjee, 2005). Além disso, a hipótese de que o citoesqueleto de actina pode funcionar como um regulador da liberação de ROS da mitocôndria (Boldogh e Pon, 2006; Gourlay e Ayscough, 2005b) reforça a idéia de um constante “cross-talk” entre causa e efeito desencadeados por insultos de Hcy.

Devido a anormalidades no sistema fosforilante citoesquelético ter sérias conseqüências na estrutura e função celular (Perrot et al., 2008) além do seu importante papel na sinalização e “cross-talk” citoesquelético (Chang e Goldman, 2004), e considerando que insultos gerados pela Hcy provocaram reorganização do citoesqueleto das células gliais

estudadas, decidimos avaliar a fosforilação dos FIs gliais em células C6. Nesse contexto, observamos que 100 e 500 μM de Hcy provocou hipofosforilação da GFAP e vimetina através de mecanismos envolvendo sinalização glutamatérgica. Esse resultado está de acordo com os resultados previamente demonstrados nos estudos *in vitro* em fatias de tecido, enfatizando a importância do sistema glutamatérgico nos efeitos neurotóxicos da Hcy.

Em resumo, o conjunto dos resultados obtidos em modelo animal mostram que a Hcy em concentrações semelhantes as encontradas em pacientes portadores de HHCY provoca alterações no sistema fosforilante associado aos FI de células neurais de maneira dependente da estrutura cerebral e da fase do desenvolvimento. Ainda, demonstramos desorganização do citoesqueleto de actina bem como alterações morfológicas em células gliais. Excitotoxicidade, estresse oxidativo e mecanismos glutamatérgicos parecem estar envolvidos nesses efeitos. Podemos supor que estes mecanismos possam agir sinergisticamente alterando a organização do citoesqueleto e levando a uma disfunção ou apoptose das células neurais. Então, o citoesqueleto pode representar um alvo na HHCY e a disfunção do citoesqueleto pode suportar, em parte, a neuropatologia desta doença. Embora as alterações produzidas pelas altas concentrações de Hcy no citoesqueleto e na morfologia celular possam ter uma função importante na neurodegeneração, os mecanismos através dos quais a desorganização das proteínas do citoesqueleto induz a disfunção neural ainda não foram determinados e a exata dimensão destas alterações como causa ou efeito da neuropatologia na HHCY precisa ser determinada.

2. CONCLUSÕES

2.1. CONCLUSÃO GERAL

Insultos com Hcy são capazes de perturbar a fosforilação das subunidades dos FIs do citoesqueleto neural, apresentando uma seletividade com relação a idade dos ratos (17 dias de idade) e a estrutura cerebral (hipocampo). Além disso, observamos efeitos antagônicos com relação às concentrações utilizadas, o que enfatiza a alta complexidade e provável *cross-talk* de vias de sinalização envolvidas.

O citoesqueleto neural mostrou-se suscetível aos efeitos crônicos da Hcy, evidenciados por alterações na expressão e imunoreatividade dos FIs estudados, e novamente, o hipocampo demonstrou-se a estrutura cerebral mais vulnerável a esses insultos.

Células glias também foram alvo para as ações da Hcy, reorganizando seu citoesqueleto e alterando o sistema fosforilante associado ao mesmo.

2.2. CONCLUSÕES ESPECÍFICAS

2.2.1 Ratos jovens submetidos ao modelo crônico de Hcy apresentaram uma marcada seletividade na alteração da expressão gênica, do conteúdo total e da fração citoesquelética das subunidades de FIs estudados, refletindo uma maior suscetibilidade do hipocampo nessas alterações. Além disso, o sistema fosforilante das duas estruturas estudadas mostrou-se insensível ao final do tratamento com Hcy.

2.2.2 O hipocampo mostrou ser mais vulnerável do que o córtex cerebral. Além disso, o hipocampo de ratos de 17 dias de idade foi sensível aos efeitos da Hcy sobre a fosforilação *in vitro* dos FIs do citoesqueleto neural. Ainda,

concentrações de Hcy referentes à HHCY moderada e grave ocasionaram efeitos antagônicos sobre esse sistema refletindo a complexidade das diversas vias de sinalização ativadas por Hcy.

2.2.3 A hiper e a hipofosforilação dos FI do citoesqueleto neural induzidas pela Hcy foram mediadas por mecanismos dependentes do influxo e *pools* intracelulares de Ca^{+2}. Além das quinases (PKCam e PKC) e fosfatases (PP2B) dependentes de Ca^{+2}, a hiperfosforilação induzida pela Hcy 100 μm foi mediada por MAPK, PI3K e PLC, enquanto a hipofosforilação desencadeada pela Hcy 500 μm foi mediada pela ativação de vias de sinalização envolvendo PP1, PP2A, mGluR e iGluR.

2.2.4 Como demonstrado com os estudos *in vitro*, os neurônios corticais não apresentaram alterações no citoesqueleto após o tratamento com Hcy. Ao contrário, os astrócitos responderam seletivamente aos insultos de Hcy, modulando o citoesqueleto de actina e GFAP, através de mecanismos envolvendo estresse oxidativo, ou induzindo apoptose celular.

2.2.5 Os FIs e MTs não sofreram modificações após o tratamento com Hcy em células C6. O citoesqueleto de actina, por sua vez, foi um importante alvo das ações da Hcy, evidenciado pelo exacerbado aumento no número de filipódios via ativação da cdc42, estresse oxidativo e mecanismos glutamatérgicos.

2.2.6 A Hcy provocou hipofosforilação da GFAP e vimetina através de mecanismos envolvendo sinalização glutamatérgica.

3. PERSPECTIVAS

Os resultados obtidos neste trabalho abrem novas possibilidades de estudos a fim de elucidar diferentes vias de sinalização envolvidas com os efeitos neurotóxicos desencadeados por altas concentrações de Hcy. Dessa maneira, nossas perspectivas são:

- Verificar o efeito da Hcy sobre a viabilidade e o citoesqueleto de neurônios e astrócitos hipocampais em cultura, bem como investigar os mecanismos de ação envolvidos;
- Avaliar os efeitos da Hcy sobre a interação neurônio-glia em co-cultura de hipocampo e córtex cerebral de ratos;
- Estudar a captação e liberação de glutamato no modelo *in vivo* e *in vitro* de homocistinúria, utilizando-se fatias de hipocampo e córtex cerebral e culturas de astrócitos e neurônios de ratos;
- Avaliar o efeito de altas concentrações de Hcy sobre a toxicidade do peptídio beta-amilóide em cultura de neurônios.

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