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ENCEFALOPATIA HEPÁTICA: ALTERAÇÕES NO METABOLISMO ENERGÉTICO, BIOSSÍNTESE DE GABA E PREJUÍZOS COMPORTAMENTAIS ESTUDADOS EM MODELOS *IN VITRO* E *IN VIVO*

Porto Alegre, Março de 2010

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Tese apresentada ao Programa de Pós-graduação em Ciências Biológicas- Bioquímica da Universidade Federal do Rio Grande do Sul, como requisito parcial para a obtenção do título de doutor em Ciências Biológicas –Bioquímica.

Orientador: Prof. Dr. Luis Valmor C. Portela.

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"Nada é impossível, a não ser que você pense que é."

Paramahansa Yogananda

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APRESENTAÇÃO

Esta tese está organizada em três **Partes**, cada uma sendo constituída dos seguintes ítens:

Parte I: Resumo, Introdução, Objetivos Geral e Específico;

Parte II: Resultados que estão apresentados na forma de **Artigos Científicos**. Cada artigo científico representa um **Capítulo** e são subdivididos em: Introdução, Materiais e Métodos, Resultados, Discussão e Referências Bibliográficas;

Parte III: Discussão, Conclusão, Perspectivas e Referências Bibliográficas citadas na Introdução da Parte I e Discussão da Parte III;

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SUMÁRIO

Parte I

RESUMO

A encefalopatia hepática (HE) é uma desordem neuropsiquiátrica que ocorre devido à falência aguda e crônica do fígado. Os mecanismos pelos quais esta doença se desenvolve não estão completamente esclarecidos, porém acredita-se que a amônia é um dos principais fatores patofisiológicos da HE. Além disso, distúrbios no sistema de neurotransmissão GABAérgico têm sido relacionado com a HE. Para estudar os efeitos da HE e da amônia sobre o SNC, desenvolvemos nesta tese um modelo de co-cultura de neurônios GABAérgicos e astrócitos provenientes camundongos que demonstrou-se bastante reproduzível, de fácil execução e representativo da interação metabólica entre neurônios e astrócitos. Também, foi verificado que a amônia exerce um papel deletério para o metabolismo energético do sistema neurotransmissor GABAérgico. As co-culturas de neurônios GABAérgicos e astrócitos quando expostos à altas concentrações de amônia, apresentaram o aumento da glicólise e da atividade do ciclo de Krebs. Além disso, foi constatado que os processos de detoxificação de amônia estavam bastante ativos, demonstrados pelo aumento das concentrações intracelulares e extracelulares de glutamina. A síntese e liberação de alanina também se encontrou aumentada, e seu papel como um agente detoxificante de amônia pode ser fundamental para os neurônios GABAérgicos. A síntese de GABA também apresentou-se alterada tanto no modelo de HE em ratos submetidos à ligação do ducto biliar (BDL), como também nas co-culturas de neurônios GABAérgicos e astrócitos expostos à amônia. Detectou-se que em ambos os modelos a síntese de GABA que envolve o ciclo de Krebs estava favorecida, quando comparada com aquela que ocorre sem o envolvimento do ciclo de Krebs. O que exatamente significa esta alteração nas rotas síntese de GABA ainda não está esclarecido, porém sabe-se que o GABA sintetizado via o ciclo de Krebs é aquele que está relacionado ao "pool" vesicular deste neurotransmissor inibitório. Empregando o modelo BDL, também estudamos os efeitos da HE sobre parâmetros comportamentais e cognitivos. Demonstramos que os animais BDL apresentam prejuízos na organização espacial e temporal das atividades locomotora e exploratória, como também na memória de curta duração para na tarefa de reconhecimento de objeto. De uma maneira geral, o trabalho desenvolvido nesta tese traz novas informações sobre os processos neuroquímicos e comportamentais envolvidos na HE que podem contribuir para o entendimento dos mecanismos patofisiológicos desta doença.

ABSTRACT

Hepatic encephalopathy (HE) is a neuropsychiatric disorder which arises due to acute or chronic liver disease. The mechanisms by which this disease develops are still not completely understood, however, it is well described that ammonia plays a pivotal role in the pathophysiology of HE. Moreover, disturbances in the GABAergic neurotransmitter system have been related to the development of HE. Aiming to study the effects of ammonia and HE in the SNC, in this thesis we standardized a model of co-culture of GABAergic neurons and astrocytes obtained from mouse. It was shown that the co-culture system was very reproducible, easy to be performed and representative of the neuronal-astrocytic interaction. The work here developed also showed that ammonia was detrimental for the energy metabolism of the GABAergic neurons and astrocytes. The co-cultures when incubated with ammonia had an increase in glycolysis and TCA cycle activity. Moreover, it was demonstrated that the detoxifying processes were highly active during hyperammonemia, i.e. increased synthesis and release of glutamine. Alanine synthesis was also increased during incubation with ammonia, and we suggest that its synthesis and release might be fundamental for the detoxification of GABAergic neurons. In another study of this thesis, we demonstrate that GABA synthesis was altered both in co-cultures of neurons and astrocytes exposed to ammonia, as well as in brain of rats with HE induced by bile duct ligation (BDL). In both systems studied, GABA synthesis via TCA cycle was favored, when compared to the one which occurs without TCA cycle involvement. To each extent this altered GABA synthesis influences the GABAergic system is not clear, however, it is known that GABA synthesized via TCA cycle is the pool related to the vesicular GABA pool. In addition, employing the BDL model, we studied the effect of HE on behavioral and cognitive parameters. The BDL rats showed impairment of spatial-temporal organization of the locomotor and exploratory activities and also in short term memory during the object memory recognition task. Therefore, the work developed during this thesis brings new information about the neurochemical and behavioral processes involved in HE, which can contribute for the understanding of the pathophysiologycal mechanisms of this disease.

LISTA DE ABREVIATURAS

ATP	Trifosfato de adenosina
BDL	Ligadura do ducto biliar
BHB	β- Hidroxibutirato
GABA	Ácido γ-aminobutírico
GABA-T	GABA aminotransferase
GAD	Glutamato descarboxilase
GAT	Transportador de alta afinidade para GABA
GDH	Glutamato desidrogenase
GS	Glutamina sintetase
HE	Encefalopatia hepática
HPLC	Cromatografia líquida de alta resolução
LC-MS	Cromatografia líquida e espectrometria de massa
NAD^+	Nicotinamida adenina dinucleotídeo oxidado
NADH	Nicotinamida adenina dinucleotídeo reduzido
NMRS	Ressonância Nuclear Magnética
PC	Piruvato Carboxilase
PDH	Piruvato Desidrogenase
PRB	Receptores Periféricos Benzodiazepínicos
SNC	Sistema Nervoso Central
SSADA	Succianto semialdeído desidrogenase

1. Introdução

1.1. Encefalopatia Hepática

1.1.1. Classificação

A Encefalopatia Hepática (HE- do inglês hepatic encephalopathy) é uma doença neuropsiquiátrica que ocorre devido à doenças agudas e crônicas do fígado (Albrecht and Jones, 1999; Hazell and Butterworth, 1999), que leva a alterações de personalidade, psicomotoras e cognitivas. Atualmente esta doença é classificada em três grandes categorias de acordo com a origem da patologia. A HE tipo A é aquela que ocorre devido à falência aguda do fígado, HE tipo B está relacionada com shunts portossitêmicos e a HE tipo C é aquela que se desenvolve a partir da cirrose (Ferenci et al., 2002; Muñoz, 2008). A encefalopatia do tipo B se distingue das demais uma vez que não há o acometimento celular hepático, sendo uma consequência de cirurgias onde se realiza anastomose portacaval ou anastomose portossistêmica intra-hepática transjugular (do inglês transjugular intrahepatic portal-systemic stent shunts – TIPS), com o objetivo de aliviar a hipertensão porta (Hazell and Butterworth, 1999).

A encefalopatia do tipo C, que é a mais comumente observada, ainda apresenta subdivisões que estão relacionadas com a duração das manifestações clínicas, sendo então subclassificada em episódica, persistente e mínima (Ferenci et al., 2002; Muñoz, 2008). A forma episódica é aquela onde o paciente apresenta distúrbios de consciência e de processos cognitivos durante curtos períodos e tempo (de horas a alguns dias), podendo ocorrer de três diferentes formas: precipitada, espontânea e recorrente. A forma precipitada se dá devido a fatores que desencadeiam a manifestação da HE, como: desidratação, sangramentos gastrintestinais, infecções e ingestão excessiva de proteínas. A espontânea é a manifestação onde não existe uma causa conhecida para o

desencadeamento da HE, uma vez que todos os fatores predisponentes foram excluídos. Por último, a recorrente é terminologia empregada quando dois episódios de HE ocorrem dentro do período de tempo de um ano. Em relação a forma persistente de HE do tipo C, neste estágio o paciente apresenta variações no padrão de consciência, porém não há o retorno para o estado de normalidade mental (Ferenci et al., 2002; Eroglu and Byrne, 2009). A HE mínima é a manifestação onde os pacientes aparentemente não apresentam sintomas característicos da HE de comprometimento do funcionamento cerebral, porém através da avaliações neuropsiquiátricas e neurofisiológicas pode-se identificar este tipo de manifestação da HE (Weissenborn et al., 2001; Ortiz, Jacas, and Córdoba, 2005; Haussinger and Schliess, 2008).

1.1.2. Manifestações clínicas

As manifestações clínicas da HE podem apresentar-se de diferentes formas, dependendo da extensão das disfunções metabólicas como também da velocidade em que a doença se desenvolve (Weissenborn et al., 2005a). Na HE decorrente de falência hepática aguda os sintomas são abruptos, podendo progredir de alterações do status mental a estupor e coma em questão de horas ou dias. Algumas vezes são observados casos de convulsões na HE tipo A, porém este sintoma pode ser uma consequência do edema cerebral (Weissenborn et al., 2005a). Já os pacientes que apresentam HE do tipo C, que se desenvolve de forma progressiva, podem apresentar uma grande variedade de manifestações clínicas, como distúrbios do sono (inversão do ciclo circadiano) e alterações de humor à prejuízos neurológicos mais graves, como letargia, desorientação e perda da consciência (Bustamante et al., 1999; Weissenborn et al., 2005a).

Desta forma, devido ao amplo espectro de sintomas, as manifestações clínicas da HE foram classificadas em 5 graus (0-4, Tabela 1), dependendo da sua gravidade (Ferenci et al., 2002; Eroglu and Byrne, 2009).

O grau zero é aquele onde não há a manifestação clara dos sintomas da HE, porém o paciente demonstra alterações no padrão do sono e alterações de comportamento que são geralmente descritas pelas pessoas que convivem com o mesmo. Estas manifestações clínicas são características da HE mínima que não é facilmente detectada por exames clínicos de rotina, necessitando exames neurofisiológicos e neuropsicológicos (Ortiz et al., 2005; Weissenborn et al., 2005a). O grau 1, onde a HE é considerada branda, é caracterizado por leve diminuição da atenção e da habilidade de realizar cálculos mentais, acompanhado de euforia ou depressão. Neste estágio também apresentam-se os primeiros sintomas de distúrbios neuromusculares como tremor e descoordenação motora (Muñoz, 2008). No grau 2, onde as manifestações clínicas são moderadas, há letargia e leve desorientação de tempo e espaço por parte do paciente, este já apresenta grande dificuldade para realizar tarefas mentais e também demonstra mudanças de personalidade e comportamento inapropriado. Nesta fase os sintomas neuromusculares são mais evidentes como o surgimento de ataxia e de asteríxis (este último também pode se manifestar no grau anterior) (Butterworth, 2000; Eroglu and Byrne, 2009). O grau 3, já se considerada HE grave, o paciente encontra-se incapaz de realizar tarefas mentais, apresenta de sonolência à semi-estupor e desorientação. Psicologicamente desenvolvem-se comportamentos bizarros e paranóia, em nível neuromuscular o asteríxis não está mais presente e há o sugirmento de reflexos hiperativos, nistagmo, rigidez e sinais positivos

de Babinski. Por fim, no grau 4, o paciente atinge o coma, com ou sem resposta ao estímulo da dor (Tabela I) (Albrecht and Jones, 1999; Muñoz, 2008).

Em relação aos problemas motores observados durante a HE, mais especificamente, tem sido descrito que os pacientes apresentam disartria, tremor, bradicinesia, hipocinesia e ataxia (Weissenborn et al., 2005a; Giewekemeyer et al., 2007). Estas deficiências motoras são mais frequentemente manifestadas na HE de grau dois, porém também podem estar presente nos graus anteriores de HE (Weissenborn et al., 2005a). A bradicinesia têm sido bastante estudada e têm sido proposto que esta manifestação clínica seja uma consequência da dificuldade do paciente em iniciar os movimentos (Joebges et al., 2003). Estas deficiências motoras são bastante graves uma vez que leva ao prejuízo na qualidade de vida do paciente com HE (Marchesini et al., 2001; Jover et al., 2005).

Tabela 1: Manifestações clínicas da Encefalopatia Hepática

Grau	Manifestações clínicas
Grau 0	Ausência de sintomas detectáveis no comportamento e personalidade
Grau 1	Distúrbios do sono, diminuição da atenção, euforia ou depressão, ansiedade Asteríxis pode estar presente
Grau 2	Letargia, leve desorientação, amnésia para eventos recentes, dificuldade para a realização de cálculos mentais Asteríxis
Grau 3	Sonolência à semi-estupor, confusão, grave desorientação, comportamento bizarro Clônus, nistagmo, sinais de Babinski
Grau 4	Coma

Além dos problemas motores observados durante a HE, muita atenção tem sido dada aos prejuízos cognitivos que estes pacientes desenvolvem. Diminuição da atenção é um sintoma bastante característico da HE, surgindo de forma lenta e progressiva da mesma forma em que o nível de consciência do paciente deteriora-se (Weissenborn et al., 2001; Amodio et al., 2005; Weissenborn et al., 2005b) . Prejuízos de memória e aprendizado também acometem os pacientes com HE (Weissenborn et al., 2005b). Porém os problemas de memória podem estar relacionados com a diminuição da atenção, pois sem a atenção necessária não há um aprendizado adequado e nem a formação de memória. Além disso, pacientes com HE também apresentam problemas de percepção visual e de orientação visual, estas deficiências por sua vez também podem interferir na memória, especialmente na memória de reconhecimento (Weissenborn et al., 2003).

A HE é um das complicações mais frequentes da cirrose hepática, estima-se que 60-80 % dos pacientes com cirrose sofrem de HE. Além disso, embora a HE do tipo C raramente leve ao óbito, o seu surgimento é sinal de mau prognóstico. Em relação aos índices de sobrevivência de pacientes cirróticos, a probabilidade de sobrevivência é de 42% e 23% depois do primeiro e terceiro ano, respectivamente, após o primeiro episódio de HE (Bustamante et al., 1999). Em relação à falência aguda fígado, a manifestação de HE também apresenta importância no prognóstico dos pacientes. As taxas de mortalidade são altas em pacientes que apresentam falência aguda do fígado, e acontecem normalmente devido ao aumento da pressão intracraniana como consequência do edema cerebral (Hazell and Butterworth, 1999). Um estudo realizado no Reino Unido demonstrou que dos 217 pacientes apresentando HE grave devido à falência aguda do fígado, 74% foram ao óbito (Eroglu and Byrne, 2009).

1.1.3. Mecanismos patofisiológicos

A teoria mais amplamente aceita no meio científico para o desenvolvimento HE é aquela que descreve a ação deletéria de certas substâncias não metabolizadas pelo fígado, como os compostos nitrogenados, para o sistema nervoso central (SNC). Estas substâncias têm origem no intestino, tanto pelo metabolismo de nutrientes da dieta como pela ação da flora intestinal, e normalmente não atingem o sistema circulatório sistêmico, pois são metabolizadas pelo fígado. No caso de prejuízos da função hepática estas substâncias atingem a circulação sistêmica e são levadas ao cérebro (Blei et al., 2001; O'Carroll, 2008). Uma vez penetrando no tecido cerebral várias mecanismos patológicos acontecem, como a disfunção de sistemas neurotransmissores, estresse oxidativo, alterações do metabolismo energético, ativação dos receptores periféricos benzodiazepínicos (PBR) e aumento da síntese de neuroesteóides (Butterworth, 2000). As substâncias neurotóxicas que são produzidas no intestino são: amônia, ácidos graxos de cadeia média e curta, fenóis e mercaptanos (Blei et al., 2001; Felipo and Butterworth, 2002). Além disso, acúmulo de magnésio também está relacionado com o desenvolvimento de HE (Mas, 2006). Dentre as substâncias anteriormente citadas, têm sido descrito que a amônia é a principal neurotoxina envolvida nos processos neuropatológicos da HE (Hazell and Butterworth, 1999; Butterworth, 2002) e este assunto será mais detalhadamente elaborado nesta tese.

O aumento das concentrações de amônia é sinal patognomônico da HE, tanto da que se origina da falência aguda como da falência a crônica do fígado. Em relação aos níveis sanguíneos, a concentração arterial de amônia encontrar-se na faixa de 0,3-0,5 mM em pacientes que apresentam falência aguda do fígado (Clemmesen et al., 1999) um aumento extremo, considerando que a faixa de normalidade se encontra entre 0,05-

0,10 μM (Felipo and Butterworth, 2002). Os pacientes com HE devido à cirrose não apresentam um aumento tão extremo nas concentrações de amônia na circulação arterial, esta se encontra na faixa de 0,1-0,2 mM, porém estes níveis são dependentes do grau de comprometimento hepático e de fatores predisponentes para a manifestação da HE. Embora o aumento das concentrações de amônia no sangue seja um achado característico de pacientes com HE, ainda há muita controvérsia a respeito de correlações entre o aumento da sua concentrações com o desenvolvimento e gravidade desta doença (Lockwood, 2004). Entretanto, alguns estudos relataram a correlação entre os níveis plasmáticos de amônia com a severidade da HE do tipo A (Clemmesen et al., 1999; Kundra et al., 2005) Aumentos significativos de amônia também foram relatados no cérebro de pacientes com HE (Felipo and Butterworth, 2002).

Os mecanismos neurotóxicos da amônia têm sido amplamente estudados, porém estes ainda não são completamente compreendidos. Como possíveis mecanismos tem-se descrito que a amônia afeta o metabolismo energético cerebral, leva ao estresse oxidativo e também à disfunção de sistemas neurotransmissores (Butterworth, 2000; Rao and Norenberg, 2001).

A respeito dos efeitos da hiperammonemia no metabolismo energético cerebral, tem sido proposta a inibição de duas enzimas chave, a piruvato desidrogenase (PDH) e a α-cetoglutarato desidrogenase, pela ação da amônia (Lai and Cooper, 1986; Zwingmann et al. 2003). Tal inibição levaria a diminuição da síntese de ATP pela fosforilação oxidativa devido à prejuízos no ciclo de Krebs (Ott et al., 2005). Como possível mecanismo compensatório para a diminuição do ciclo de Krebs, aconteceria o aumento da glicólise, e esta situação já foi descrita em diferentes estudos (Ratnakumari and Murthy, 1992; Hertz and Kala, 2007; Johansen et al., 2007). Entretanto, a literatura

que abrange o efeito da hiperamonemia sobre o metabolismo energético cerebral é bastante contraditória, possivelmente devido à variedade de modelos experimentais empregados (Rao and Norenberg, 2001). Porém, recentemente foi demonstrado que culturas de neurônios cerebelares quando expostos à altas concentrações de amônia não resultava na diminuição ou inibição do ciclo de Krebs como anteriormente proposto. Ao contrário, este estudo descreve a ativação de ambos ciclo de Krebs e glicólise durante a exposição à amônia (Johansen et al., 2007).

O cérebro, ao contrário do fígado, não dispõe do ciclo da uréia para o processo de detoxificação de amônia através da conversão desta neurotoxina no metabólito não tóxico ao organismo humano. O principal mecanismo para a detoxificação da amônia no cérebro se dá pela atividade da enzima glutamina sintetase (GS), que é seletivamente expressa em astrócitos (Norenberg and Martinez-Hernandez, 1979). Não é surpreendente o fato de que níveis aumentados de glutamina são frequentemente encontrados durante processos de hiperamonemia. Pacientes com HE apresentam níveis aumentados de glutamina tanto no soro como em líquor (Zwingmann and Butterworth, 2005; Albrecht and Norenberg, 2006; Jayakumar et al., 2006). Além disso, o aumento da glutamina também tem sido relacionado com os processos patofisiológicos da HE, uma vez estando relacionada com indução de espécies reativas de oxigênio e disfunção mitocondrial (Albrecht and Zielińska, 2002; Jayakumar et al., 2004; Jayakumar et al., 2006). A enzima glutamato desidrogenase (GDH), que é expressa em ambos astrócitos e neurônios, também age como detoxificante de amônia através da reação de síntese de glutamato a partir do α-cetoglutarato por aminação redutiva (Yudkoff et al., 1990; Ott et al., 2005; Schousboe and Waagepetersen, 2007). Outra reação que também acredita-

se participar da detoxificação da amônia é a síntese de alanina através da enzima alanina aminotrasnferase (ALAT) (Zwingmann and Leibfritz, 2005).

O estresse oxidativo tem sido relacionado com a neurotoxicidade da amônia e há evidências mostrando que esta neurotoxina leva ao aumento da produção de radicais livres e diminui a ação de várias enzimas antioxidantes tanto em estudos *in vivo* como *in vitro* (Kosenko et al., 1997; Murthy et al., 2001; Norenberg, 2003; Norenberg et al., 2004). Estudos realizados em ratos que receberam injeções de acetato de amônia demonstraram que a hiperamonemia leva ao aumento do radical superóxido e também à diminuição da atividade da glutationa peroxidase, superóxido dismutase e catalase (Kosenko et al., 1997). Uma das consequências do estresse oxidativo é a disfunção mitocondrial que pode acontecer devido à indução da permeabilidade mitocondrial transitória, que consequentemente pode acarretar no colapso do potencial de membrana desta organela, levando ao inchaço da matriz mitocondrial e prejuízos no funcionamento da cadeia fosforilativa e síntese de ATP (Norenberg et al., 2004; Rao et al., 2005). Estes efeitos de inchaço e disfunção celular já foram descritos em culturas celulares de astrócitos expostas a concentrações tóxicas de amônia, e a turgescência astrocitária está relacionada com o edema cerebral observado na HE (Bai et al., 2001; Albrecht and Norenberg, 2006).

Como dito anteriormente, um dos mecanismos patofisiológicos na HE é o distúrbio de sistemas neurotransmissores. Distúrbios nos sistemas neurotransmissores glutamatérgico, GABAérgico, serotonérgico, monoaminérgico e histaminérgico já foram descritos (Butterworth, 2000).

A hipótese de aumento do tônus do sistema GABAérgico na HE foi sugerido no início da década de 80 quando experimentos foram realizados no modelo de falência

aguda do fígado induzido pela galactosamina em coelhos, que demonstrou que o padrão de respostas do potencial evocado eram semelhantes ao de animais que eram tratados com agonistas dos receptores GABAérgicos (Jones and Basile, 1998; Ahboucha and Butterworth, 2004). Além disso, foi constatada que a administração de flumazenil, um antagonista do sítio benzodiazepínico do receptor de $GABA_A$, era benéfico para pacientes com HE (Ahboucha and Butterworth, 2004). Estes autores propuseram a teoria do aumento do tônus do sistema GABAérgico na HE, que resultava do aumento da neuroinibição. Depois destas primeiras evidências, vários estudos foram realizados no intuito de elucidar os mecanismos envolvidos no aumento do tônus GABAérgico.

O aumento do tônus GABAérgico poderia ser consequente das seguintes alterações no sistema GABAérgico: aumento da síntese e liberação do neurotransmissor GABA, alterações na integridade do receptor $GABA_A$, alterações no sistema de captação de GABA, aumento da síntese de substâncias endógenas que são moduladoras do sistema GABAérgico. Até o presente momento, a literatura descreve que os níveis cerebrais de GABA estão inalterados tanto em pacientes com HE de origem aguda como crônica (Albrecht and Zielińska, 2002; Ahboucha and Butterworth, 2004). Estudos realizados em diferentes modelos animais de falência hepática aguda e crônica também descreveram que as concentrações de GABA estavam inalteradas no SNC (Zwingmann et al., 2003; Ahboucha and Butterworth, 2004).

A respeito da integridade dos receptores de GABA, vários estudos demonstraram que os receptores $GABA_A$ têm sua integridade inalterada na HE (Ahboucha et al., 2003; Ahboucha and Butterworth, 2005). Alguns estudos se detiveram em verificar se não existia o aumento da densidade dos receptores benzodiazepínicos, uma vez que antagonistas deste sítio eram benéficos para uma

população de pacientes com HE. Apenas dois estudos demonstraram o aumento destes sítios, porém muitos outros estudos realizados posteriormente não demonstraram alterações na densidade dos receptores benzodiazepínicos (Ahboucha and Butterworth, 2005). Além disso, também foi descrita a conservação da densidade e afinidade do receptor $GABA_A$ para o seu neurotransmissor GABA (Ahboucha et al., 2003). Porém isto não significa que o receptor como um todo não tenha alterações na regulação da sua atividade. Isto se deve ao fato de que as diferentes subunidades que compõem o receptor $GABA_A$ interagem uma com as outras, e a expressão de certas subunidades pode acarretar numa maior ou menor afinidade do receptor com os seus moduladores. Sabe-se que os agonistas benzodiazepínicos modulam positivamente a neurotransmissão mediada pelo receptor GABA, como também os neuroesteróides são agonistas dos efeitos de ambos GABA e benzodiazepínicos (Figura 1) (Ahboucha and Butterworth, 2005). Logo, mudanças na interação entre estas subunidades poderiam também levar ao aumento do tônus GABAérgico na HE. Entretanto, um estudo com tecido cerebral *postmortem* de pacientes com HE revelou que as interações entre os sítios de ligação de GABA, benzodiazepínicos e neuroesteróides estavam conservadas (Ahboucha et al., 2003). Porém este estudo foi realizado apenas com tecido originário do córtex prefrontal e os ensaios interação entre os sítios ativos do receptor $GABA_A$ foram realizados através de "binding" com agonistas e antagonistas deste receptor. Recentemente, um estudo realizado em ratos com HE tipo A e utilizando a tecnologia de real time – PCR, detectou alterações na expressão das subunidades α_1, β_1 e γ_2 em diferentes estruturas cerebrais. Logo, as alterações observadas poderiam estar relacionadas com a expressão de receptores $GABA_A$ com afinidades diferentes para os seus ligantes, levando à alterações na neurotransmissão GABAérgica (Li et al., 2005).

A amônia também age diretamente no receptor $GABA_A$, aumentando a ativação deste receptor pelos seus ligantes (Basile, 2002). Estudos realizados em culturas de neurônios mostraram que a amônia potencia as correntes de cloreto dos receptores $GABA_A$ (Takahashi et al., 1993). Além disso, concentrações moderadas de amônia aumentam a afinidade de agonistas do receptor $GABA_A$ em cérebro de ratos (Ha and Basile, 1996).

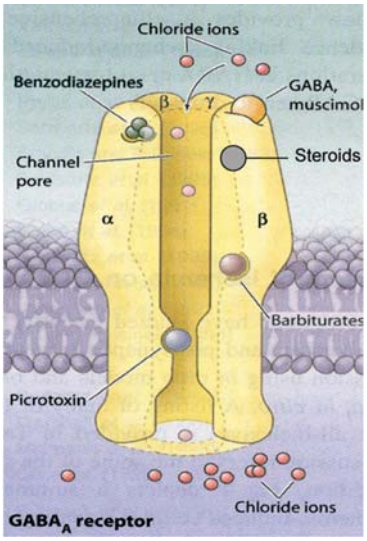

Figura 1. **Sítios de ligação do receptor $GABA_A$**: As subunidades do receptor $GABA_A$ apresenta sítios específicos para: neurotransmissor GABA, benzodiazepínicos, picrotoxina e neuroesteróides (Fonte: Ahboucha e Butterworth, 2005).

Além dos efeitos observados sobre o receptor $GABA_A$, um estudo demonstrou a diminuição da ligação do GABA nos receptores $GABA_B$ em córtex de ratos com HE (Oja et al.,1993). Esta diminuição poderia levar ao aumento da liberação de GABA das vesículas sinápticas, uma vez que o receptor $GABA_B$ localizado na pré-sinapse é responsável pela inibição da despolarização que leva à liberação do neurotransmissor

GABA. De fato, o mesmo grupo de autores observou o aumento da liberação de GABA em fatias de córtex cerebral de ratos com HE (Wysmyk et al., 1992).

O efeito da amônia sobre o sistema GABAérgico também foi descrito sobre a atividade dos transportadores de GABA astrocitários. No estudo de Bender e Norenberg (2000) foi demonstrado a diminuição da captação de GABA em cultura de astrócitos, que consequentemente levaria ao aumento das concentrações do neurotransmissor na fenda sináptica. Além disso, um estudo realizado em ratos com HE também demosntrou a diminuição de sítios de ligação do $GABA_B$ em córtex cerebral (Oja et al., 1993). Porém, como dito anteriormente, as concentrações de GABA apresentam-se inalteradas na HE. Entretanto, deve-se considerar o fato que várias das medidas das concentrações do neurotransmissor GABA foram realizadas em amostras de tecido homogeneizado, assim como no fluído de microdiálise. Logo, deve-se considerar o fato que estas dosagens podem não verdadeiramente representar as concentrações de GABA da fenda sináptica.

O aumento da síntese de neuroesteróides também está relacionado com o aumento do tônus GABAérgico. Certos neuroesteróides, como a alopregnanolona tetrahidroprogesterona, são potentes agonistas do receptor $GABA_A$ pois eles aumentam a duração e frequência de abertura do canal de cloreto do receptor que consequentemente leva ao aumento da neuroinibição mediada pelo GABA (Ahboucha and Butterworth, 2008). Os neuroesteróides são sintetizados posteriormente ao transporte do colesterol do citosol para dentro da membrana interna mitocondrial, através da ativação do receptor mitocondrial do tipo benzodiazepínico (receptor periférico para benzodiazepínicos – PRB, também conhecido como proteína translocadora) (Ahboucha and Butterworth, 2007; Ahboucha and Butterworth, 2008).

O aumento da síntese destes compostos foi encontrada em modelos animais assim como em pacientes com HE (Ahboucha and Butterworth, 2007; Ahboucha and Butterworth, 2008). O mecanismo proposto para o aumento da síntese destes compostos é relacionado com o aumento da expressão dos PRB, que é localizado principalmente em astrócitos. Vários estudos demonstraram o aumento do PRB em pacientes com HE como também em modelos animais de HE (Jones, 2002; Ahboucha and Butterworth, 2007). Além disso, foi demonstrado que a amônia induz o aumento da expressão de PRB, levando indiretamente ao aumento da síntese de neuroesteróides que ativam o receptor $GABA_A$ (Albrecht and Jones, 1999; Ahboucha and Butterworth, 2007).

1.1.4. Tratamento

O tratamento da HE se dá pela primeiramente pela identificação dos fatores predisponentes que levaram à manifestação da HE, como também da gravidade do comprometimento hepático. Os fatores predisponentes à manifestação da HE, como desidratação, diarréia, sangramentos gastrintestinais, aumento de ingestão de proteínas, entre outros, são normalmente fáceis de corrigir e algumas vezes não é necessário a intervenção farmacológica (Muñoz, 2008). Porém, muitas vezes a terapia farmacológica é necessária e um dos principais objetivos destas terapias é a redução de amônia que chega à circulação sanguínea sistêmica por fármacos que diminuem a sua produção ou aumentem a sua excreção (Mas, 2006). Entre estes fármacos encontram-se os dissacarídeos não absorvíveis, como a lactulose, que leva à acidificação do cólon intestinal e consequentemente ao efeito catártico, aumentando a eliminação de amônia (Blei et al., 2001). A terapia com antibióticos, como a neomicina e metranidazol,

também são utilizados com o propósito de inibir a produção de amônia pelas bactérias intestinais (Muñoz, 2008). Além disso, outros fármacos podem ser utilizados para aliviar e/ou reverter os sintomas da HE, como os benzodiazepínicos (Eroglu and Byrne, 2009). No caso de HE persistente ou episódica em pacientes com cirrose, deve-se considerar a possibilidade de transplante de fígado, uma vez que estes pacientes apresentam baixas taxas de sobrevivência (O'Carroll, 2008; Eroglu and Byrne, 2009).

1.1.5. Modelos experimentais para o estudo da HE

Diversos modelos experimentais têm sido utilizados para o estudo da HE, empregando tanto modelos experimentais *in vivo* como *in vitro*. Estudos realizados em *in vitro* basicamente utilizam culturas celulares de astrócitos, neurônios e outros tipos celulares que são expostos à concentrações elevadas de amônia e outros compostos que também estão relacionados com a HE (Butterworth et al., 2009). Os modelos experimentais para o estudo *in vivo* da HE podem ser divididos em dois grandes grupos, aqueles que estudam a HE devido a falência hepática aguda ou crônica (Butterworth et al., 2009; Tuñón, 2009). Estes são subdivididos em dois grupos, de acordo com o tipo de indução ao dano hepático, que pode ser realizado tanto por processos cirúrgicos como pela administração de substâncias químicas tóxicas que lesam o fígado (Tabela 2). Diversas espécies já foram utilizadas para o estudo *in vivo* da HE, como ratos, cães, coelhos e porcos, porém os modelos realizados em ratos são os mais comumente observados.

Nesta tese daremos ênfase para os estudos *in vitro* em co-culturas de neurônios e astrócitos, como também cultura de neurônios expostos à amônia, como também para

o modelo *in vivo* de HE do tipo C induzida pelo processo cirúrgico de ligação do ducto biliar (do inglês bile duct ligated -BDL) em ratos.

Tabela 2. Modelos animais de encefalopatia hepática

Tipo A		Tipo B e C	
Cirúrgico	Desvascularização hepática	Cirúrgico	Ligação do ducto biliar
	Hepatectomia		Portacaval anastomose
	Portacaval anastomose + amônia		
Químico	Galactosamina	Químico	Tioacetamida
	Acetoaminofeno		
	Tioacetamida		

1.2. Sistema neurotransmissor GABAaérgico

O ácido γ-aminobutírico (GABA) é o mais abundante neurotransmissor inibitório do SNC. Este neurotransmissor é fundamental em diversos processos cerebrais, estando envolvido em processos como cognição, atividade motora e ciclo cicardiano (Olsen & Betz, 2006). Alterações neste sistema neurotransmissor têm sido relacionado com diversas patologias, como a epilepsia e esquizofrenia (Olsen & Betz, 2006).

A síntese do neurotransmissor GABA ocorre principalmente a partir da glicose. Esta é metabolizada no ciclo de Krebs até α-cetoglutarato que é subsequentemente convertido à glutamato, reação que pode ocorrer pela atividade das enzimas GDH, aspartato amino transferase e outras transmaminases (Schousboe and Waagepetersen, 2006). O glutamato é então descarboxilado através da enzima L-glutamate decarboxilase (GAD), reação dependente do cofator piridoxal fosfato, gerando a molécula de GABA (Schousboe and Waagepetersen, 2006). A GAD é considerada um

marcador de neurônios GABAérgicos do SNC, entretanto alguns estudos já demonstraram que a GAD também pode estar presente em uma pequena subpopulação de neurônios excitatórios (Sonnewald et al., 2006). A enzima GAD apresenta-se na forma de duas isoformas que são abundantemente expressas no SNC, a GAD_{65} e a GAD_{67}, que se diferenciam pela sua localização e propriedades regulatórias. A GAD_{67} é aquela que é presente no citosol dos neurônios GABAérgicos, tanto no corpo celular como nos seus processos (Stone et al., 1999). Já a GAD_{65} é predominantemente encontrada nos terminais nervosos e possivelmente se encontra ancorada ou associada à membrana das vesículas sinápticas que contém o neurotransmissor (Waagepetersen et al. 2001; Tian et al. 1999). A metabolização do GABA ocorre pela atividade das enzimas GABA aminotransferase (GABA-T) e pela succinato semialdeído desidrogenase (SSADH), conhecidas como o GABA "shunt". A enzima GABA-T, que é localizada na matriz mitocondrial e é expressa abundantemente no SNC, converte GABA à succinato semialdeído, enquanto glutamato é formado a partir do α-cetoglutarato. O succinato semialdeído é subseqüentemente oxidado à succinato que entra no ciclo de Krebs (Figura 2) (Waagepetersen et al., 1999a).

A sinalização inibitória do GABA se dá através da ligação deste neurotransmissor no seus receptores $GABA_A$ e $GABA_B$. O receptor $GABA_A$ é um receptor ionotrópico formado por proteínas heteropentaméricas que são constituídas por subunidades. Até o presente momento foram identificadas 18 diferentes subunidades para o receptor $GABA_A$, entre elas: α (1-6), β (1-3), γ (1-3), δ, ε (1-3), π e θ (Owens and Kriegstein, 2002). Esta grande variedade de subunidades confere ao receptor uma grande variedade de possíveis combinações, entretanto certas combinações são mais encontradas, como a $\alpha1\beta2\gamma2$ (Jacob et al., 2008). Normalmente os receptores $GABA_A$

contém pelo menos uma subunidade α , β e γ, esta última podendo ser substituída por δ, ε, π ou θ (Figura 3) (Owens and Kriegstein, 2002).

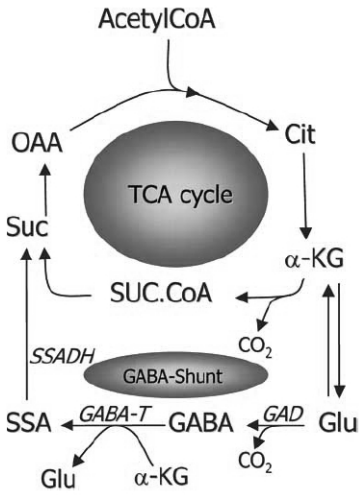

Figura 2: Representação esquemática do GABA "shunt" e ciclo de Krebs. Abreviações: aKG: α-cetuglutarato; SSADH: suscinato semialdeído; GABA-T: GABA transaminase; GAD: glutamato descarboxilase. (Fonte: Waagepetersen & Schousboe, 2006).

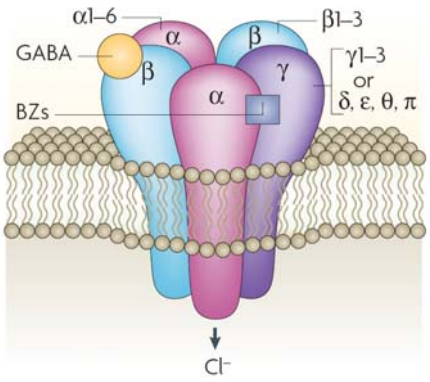

Figura 3. Subunidades do receptor $GABA_A$: O receptor GABA e as suas subunidades constituintes e sítios para a ligação do neurotransmissor inibitório e benzodiazepínicos. (Fonte: Jacob et al., 2008).

A ligação do GABA no receptor $GABA_A$ leva ao influxo de íons cloreto para o meio intracelular do neurônio pós-sináptico, hiperpolarizando-o. Este receptor também apresenta outros sítios de ligação, como para barbitúricos, benzodiazepínicos e neuroesteóides, como descrito anteriormente (Figura 1). O receptor $GABA_B$, do tipo metabotrópico, está ligado à proteína G e é composto por duas subunidades, constituindo as isoformas $GABA_{B1a-b}$ e $GABA_{B2}$ (Ulrich and Bettler, 2007). Estes receptores medeiam a inibição tanto na pós como na pré–sinapse. Este processo pode ocorrer tanto pela abertura de um canal para o íon potássio como também pela menor condutância do íon cálcio. Este último é o processo de inibição que ocorre na pré-sinapse, diminuindo a liberação de neurotransmissores (Ulrich & Bettler, 2007; Olsen & Betz, 2006).

O GABA é liberado das vesículas sinápticas da pré-sinapse através da depolarização dependente de cálcio. Entretanto, estudos demonstraram a liberação de GABA não vesicular pode ocorrer em células neuronais e não neuronais através do transporte reverso de GABA (Treiman 2001; Schousboe and Waagepetersen, 2006).

A neurotransmissão do GABA é terminada pela ação de trasportadores de alta afinidade para GABA (GAT), localizados nos terminais nervosos da pré-sinapse como também nos astrócitos da fenda sináptica (Schousboe and Waagepetersen, 2006). Até o presente momento foram clonados e caracterizados quatro transportadores de GABA, GAT1-4 (nomenclatura empregada para os transportadores clonados em camundongos) (Schousboe, 2003; Schousboe et al., 2004). Entre os transportadores de GABA, GAT1 é o mais abundantemente expresso e apresenta-se localizado preferencialmente nos segmentos axonais de neurônios GABAérgicos (Schousboe and Kanner, 2002) (Figura 4). Os outros transportadores estão mais ubiquamente distribuídos, entretanto GAT4

parece ser preferencialmente expresso nos processos dos astrócitos que estão em contato com neurônios GABAérgicos. Desta forma, os transportadores GAT1 e GAT4 medeiam a captação de GABA e consequentemente o término da neurotransmissão GABAérgica (Schousboe et al., 2004).

A maior parte do GABA é captado pelos neurônios da pré-sinapse para ser reutilizado como neurotransmissor. O GABA captado pelos astrócitos pode ser metabolizado pela GABA-T e ciclo de Krebs à CO_2 ou pode ser convertido a glutamina (Bak et al., 2006). A glutamina pode então ser transferida para os neurônios GABAérgicos para subsequentemente ser convertida à GABA pela ação da glutaminase fosfato dependente e GAD. Este processo é conhecido como ciclo do GABA-glutamato-glutamina e é muito importante para a manutenção do pool neurotransmissor e para a transferência de nitrogênio (Bak et al., 2006).

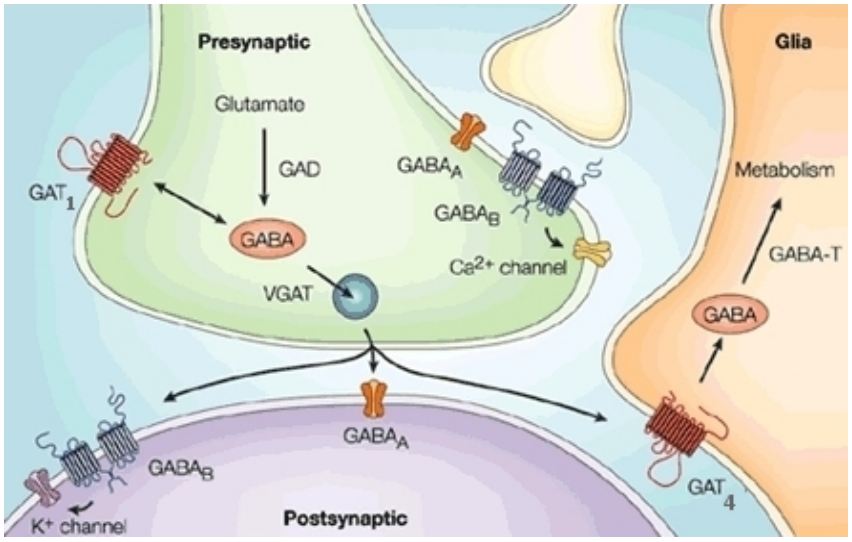

Figura 4. Representação da sinapse GABAérgica: (Adaptado de: Jacob et al., 2008).

2. Objetivo

2.1. Objetivo geral

Estudar em culturas GABAérgicas os efeitos da amônia sobre parâmetros de detoxificação, metabolismo energético e síntese de GABA, como também verificar em um modelo *in vivo* de HE os efeitos desta patologia sobre a síntese de GABA e sobre aspectos comportamentais.

2.2. Objetivos específicos

2.2.1. Padronizar um método de co-cultura de neurônios e astrócitos do sistema GABAérgico que seja de fácil realização e que permita o estudo da interação neurônio-astrócito em um sistema *in vitro*.

2.2.2. Estudar em co-culturas de neurônios e astrócitos GABAérgicos como também em culturas de neurônios GABAérgicos os processos de detoxificação de amônia como também os efeitos da amônia sobre parâmetros do metabolismo energético.

2.2.3. Estudar os efeitos da HE e das concentrações tóxicas de amônia nas rotas metabólicas de síntese de GABA em cérebro de ratos BDL e em co-culturas de neurônios e astrócitos GABAérgicos.

2.2.4. Estudar em ratos BDL, com HE do tipo C, as atividades locomotora e exploratória, assim como a memória de reconhecimento de objeto.

Parte II

CAPÍTULO I

Demonstration of Neuron-Glia Transfer of Precursors for Gaba Biosynthesis in a Co-Culture System of Dissociated Mouse Cerebral Cortex

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ORIGINAL PAPER

Demonstration of Neuron-Glia Transfer of Precursors for Gaba Biosynthesis in a Co-Culture System of Dissociated Mouse Cerebral Cortex

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Abstract Co-cultures of neurons and astrocytes were prepared from dissociated embryonic mouse cerebral cortex and cultured for 7 days. To investigate if these cultures may serve as a functional model system to study neuronglia interaction with regard to GABA biosynthesis, the cells were incubated either in media containing [U-^{13}C] glutamine (0.1, 0.3 and 0.5 mM) or 1 mM acetate plus 2.5 mM glucose plus 1 mM lactate. In the latter case one of the 3 substrates was uniformly ^{13}C labeled. Cellular contents and ^{13}C labeling of glutamate, GABA, aspartate and glutamine were determined in the cells after an incubation period of 2.5 h. The GABA biosynthetic machinery exhibited the expected complexity with regard to metabolic compartmentation and involvement of TCA cycle activity as seen in other culture systems containing GABAergic neurons. Metabolism of acetate clearly demonstrated glial synthesis of glutamine and its transfer to the neuronal compartment. It is concluded that this co-culture system serves as a reliable model in which functional and pharmacological aspects of GABA biosynthesis can be investigated.

Keywords Glutamate · Glutamine · Aspartate · ^{13}C · Metabolism · Neurotransmission

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Introduction

Classical studies of GABA biosynthesis in the brain or in different brain tissue preparations such as brain slices using radiolabeled precursors, have demonstrated that label from glucose and lactate is incorporated into GABA directly in the neuronal compartment whereas acetate leads to label indirectly via transfer of glutamine synthesized from acetate in the glial compartment [1]. This latter metabolic relation between neurons and glial cells has also been found in a co-culture system consisting of cerebral cortical neurons maintained on a preformed layer of confluent astrocytes [2]. Subsequent studies of GABA biosynthesis in cultured cerebral cortical neurons in which proliferation of astrocytes was prevented using a cytostatic agent have additionally revealed that GABA biosynthesis from glutamine to a considerable extent requires the participation of the tricarboxylic acid (TCA) cycle [3, 4].

As the GABA neurotransmission system constitutes an important target for numerous drugs (see [5]) it is important to have a simple in vitro model system in which GABA biosynthesis can be studied under conditions in which the neuron-glial metabolic interaction remains intact. The previously developed co-culture system [2] did fulfill these specifications but it has turned out to be rather time consuming to work with, particularly due to the long tissue culture period. An analogous co-culture system simply consisting of a mixed culture of dissociated fetal cerebral cortex in which astrocytic proliferation was allowed to proceed was used to demonstrate neuron-glial signalling mechanisms involved in regulation of expression of astroglial glutamate transporters GLAST and GLT-1 [6]. This system might therefore additionally be of use for studies of metabolic interactions between neurons and astrocytes. The present study was therefore undertaken to

characterize the metabolic pathways for GABA biosynthesis using this culture system in combination with ^{13}C-labeled precursors such as [U-^{13}C]glutamine, [U-^{13}C]glucose, [U-^{13}C]lactate and [U-^{13}C]acetate and LC-MS technology to monitor labeling of GABA and its related amino acids glutamate, aspartate and glutamine. Analogous studies have recently successfully been performed in other systems of brain cell cultures [3, 4, 7] which allows a comparison between the previous work and the present more simple and rapid system to be performed.

Experimental Procedures

Materials and Methods

Materials

Seven-day-old NMRI mice were obtained from Taconic M&B (Ry, Denmark). Plastic tissue culture dishes were purchased from NUNC A/S (Roskilde, Denmark), fetal bovine serum from GIBCO, Invitrogen (Taastrup, Denmark). Culture medium and poly-D-lysine (MW > 300,000) were from Sigma Chemical Co. (St. Louis, MO, USA). Penicillin was from Leo (Ballerup, Denmark). Isotopically labeled compounds were either from Cambridge Isotopes Laboratories, Inc. (Massachusetts, USA) or Isotec (a subsidiary of Sigma Chemical Co.). All other chemicals used were of the purest grade available from regular commercial sources.

Co-Cultures of Cortical Neurons and Astrocytes

To obtain co-cultures of cortical neurons and astrocytes, the method described by Hertz et al. [8] to prepare pure neuronal culture was used, with the exception that cytosine arabinoside which is used to prevent astrocytic proliferation, was not added to this cell preparation. Consequently, astrocytes proliferate and interact with the neurons. Briefly, cerebral cortices were removed from 15 gestation day mouse fetuses and exposed to trypsinization (0.25 mg/ml trypsin, 15 min, 37°C). Subsequently, the tissue was triturated in a DNase solution (75 i.u/ml) containing a trypsin inhibitor (0.53 mg/ml) from soybeans. The cells were suspended (2.75×10^6 cells/ml) in a slightly modified Dulbecco's medium [9] containing 19 mM KCl, 31 mM glucose, 0.2 mM L-glutamine, 7 μM *p*-aminobenzoate, 26.2 mM $NaHCO_3$, 50,000 i.u. penicillin and 10% (v/v) fetal bovine serum. The cells were cultured in poly-D-lysine coated 6 well plates (2 ml/well) for 7–8 days, a period during which they exhibited an abundant proliferation of astrocytes as well as neuronal migration as observed by light microscopy.

Incubation Experiments

At 7–8 days in vitro, the medium was removed and the cultures were rinsed twice in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 7.3 mM Na_2HPO_4, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, pH 7.4, 37°C) and incubated for two and a half hours in a serum free medium (2 ml/well) containing one of the following 6 different incubation media: (i) 3 mM [U-^{13}C]acetate, 2.5 mM glucose and 1 mM lactate; (ii) 3 mM acetate, 2.5 mM [U-^{13}C]glucose and 1 mM lactate; (iii) 3 mM acetate, 2.5 mM glucose and 1 mM [U-^{13}C]lactate; (iv) 0.1 mM [U-^{13}C]glutamine, 2.5 mM glucose and 1 mM lactate; (v) 0.3 mM [U-^{13}C]glutamine, 2.5 mM glucose and 1 mM lactate or (vi) 0.5 mM [U-^{13}C]glutamine, 2.5 mM glucose and 1 mM lactate. The incubation period was terminated by removing the medium from the cells which were subsequently rinsed twice with ice-cold PBS to ensure complete removal of the incubation medium. Subsequently, 70% (v/v) ice-cold ethanol was added to the culture plate and the cells were scraped off and the cell-ethanol mixture centrifuged (20,000 *g*, 20 min, 4°C) to separate the soluble extract from the insoluble components. Both the incubation media and the cell extracts were lyophilized and reconstituted in water for biochemical analyses.

Biochemical Analyses

Amino acids were separated and quantified by reversed-phase HPLC employing pre-column, on-line *o*-phthaldialdehyde derivatization and fluorescence detection (excitation 350 nm; detection 450 nm) as described by Geddes and Wood [10]. The Phenomenex EZ:faast amino acid analysis kit for LC-MS was used for analysis of labeling in relevant amino acids. Mass spectrometric analysis was performed on an LC-MS system consisting of a Shimadzu LCMS-2010 mass spectrometer coupled to a Shimadzu 10A VP HPLC system. Protein content was determined according to Lowry et al. [11] using bovine serum albumin as the standard.

Data Analysis

Data analysis was performed employing Microsoft Excel 2003 and GraphPad Prism v4.01 softwares. All labeling data were corrected for natural abundance of ^{13}C by subtracting the mass distribution of a standard containing the relevant metabolites. Isotopic enrichment was calculated according to Biemann [12]. All data are presented as averages ± the standard error of the mean (S.E.M.) and differences between groups were analyzed statistically using one-way ANOVA followed by the Tukey post hoc

test. A P-value < 0.05 was considered statistically significant. The average percent of ^{13}C labeled carbon atoms, i.e. the percent molecular carbon labeling (MCL), was calculated as initially introduced by Bak et al. [13] in intracellular glutamate, GABA, aspartate and glutamine.

Results

Amino Acid Contents

The co-cultures of neocortical neurons and astrocytes were incubated in media containing a mixture of 3.0 mM acetate, 2.5 mM glucose, 1.0 mM lactate or 0.1, 0.3 or 0.5 mM glutamine together with 2.5 mM glucose and 1.0 mM lactate. Cellular contents of the amino acids glutamate, GABA, aspartate and glutamine were determined and the results presented in Fig. 1A–D. It is seen that subsequent to incubation in the medium containing acetate, glucose and lactate, the contents of GABA, aspartate and glutamine amounting to about 10–20 nmol $\times$ mg^{-1} protein were approximately one-third of that of glutamate. When the cultures were incubated in the media containing glucose and lactate together with glutamine at increasing concentrations, the contents of the four amino acids were higher compared to the acetate containing medium (Fig. 1A–D). Moreover, in case of glutamine the cellular content increased as a function of the glutamine concentration in the incubation medium (Fig. 1A). In contrast to this, the glutamate and aspartate contents were not affected by the glutamine concentration. However, in the case of GABA, the intracellular content exhibited a decrease at the extracellular glutamine concentrations of 0.3 and 0.5 mM compared to 0.1 mM (Fig. 1B).

Metabolism of [U-^{13}C]glutamine

In order to investigate the capacity for glutamine uptake and metabolism, the co-cultures were incubated for 2.5 h in medium containing [U-^{13}C]glutamine at concentrations of 0.1, 0.3 and 0.5 mM. Subsequently, the labeling in intracellular and extracellular glutamine was determined using LC-MS. Figure 2 shows that the total percent labeling (MCL) in intracellular glutamine increased concentration dependently in a saturable fashion reaching a level of approx. 70% at the highest extracellular glutamine concentration. Figure 3 shows that the amino acids glutamate, GABA and aspartate were also labeled from glutamine in a concentration dependent manner. Regardless of the glutamine concentration, labeling in glutamate exceeded that of GABA and aspartate, and aspartate labeling was higher than that of GABA except at the lowest extracellular glutamine concentration (0.1 mM) where the MCL was essentially the same for the two amino acids.

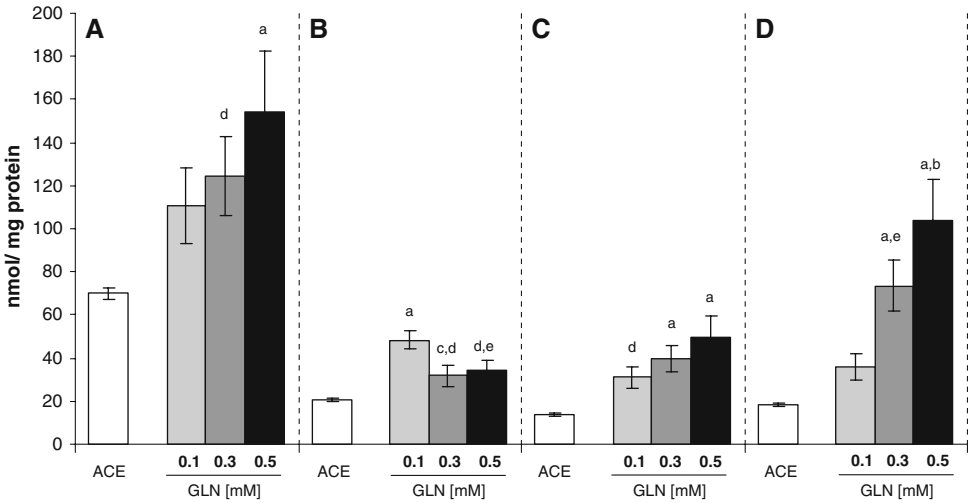

Fig. 1 Contents (nmol $\times$ mg^{-1} protein) of glutamate, GABA, aspartate and glutamine in cultures of dissociated mouse cerebral cortex. Cultures were prepared as described in "Experimental Procedures" and subsequently incubated with 3 mM acetate, 2.5 mM glucose and 1 mM lactate (white bars); 0.1 mM glutamine (light grey bars); 0.3 mM glutamine (dark gray bars) or 0.5 mM glutamine (black bars) together with 1 mM lactate and 2.5 mM glucose to investigate the amino acid contents. Results are presented as averages $\pm$ S.E.M. for intracellular glutamate (A), GABA (B), aspartate (C) and glutamine (D) representing 14 cultures for the acetate protocol and 4–5 cultures for the glutamine protocol. The statistical analysis was performed using one way ANOVA followed by the Tukey post hoc test (Experimental procedures) and the small letters represent the following: (a) significantly different from the acetate group ($P < 0.001$); (b) significantly different from the 0.1 mM glutamine group ($P < 0.001$); (c) significantly different from the 0.1 mM glutamine group ($P < 0.01$); (d) significantly different from the acetate group ($P < 0.05$) and (e) significantly different from the 0.1 mM glutamine group ($P < 0.05$)

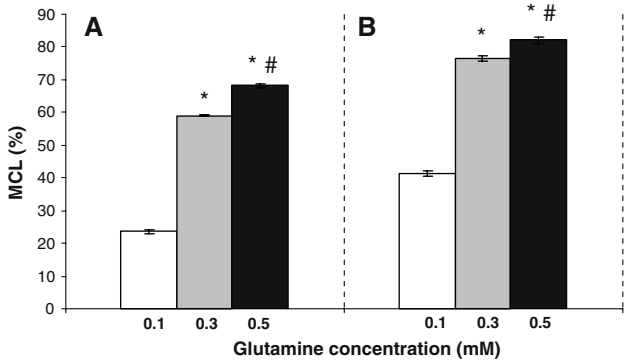

Fig. 2 Average incorporation of ^{13}C (MCL, %) in intracellular (A) and extracellular (B) glutamine in cultures of dissociated mouse cerebral cortex. Cultures were prepared as described in "Experimental Procedures" and subsequently incubated for 2.5 h in media containing [U-^{13}C]glutamine at concentrations of 0.1 mM (white bars), 0.3 mM (grey bars) and 0.5 mM (black bars). The molecular carbon labeling (MCL) values were determined in cell extracts as detailed in "Experimental Procedures". Results are averages ± S.E.M. of 4–5 cultures. An asterisk indicates statistically significant differences determined by ANOVA (see "Experimental Procedures") from 0.1 mM glutamine ($P < 0.001$) and a number sign from 0.3 mM glutamine by ($P < 0.001$)

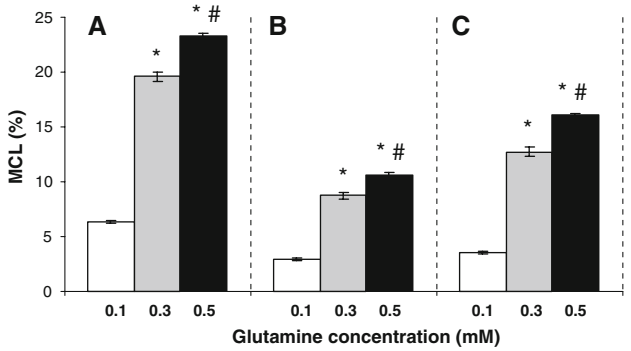

Fig. 3 Average incorporation of ^{13}C (MCL, %) in glutamate (A), GABA (B) and aspartate (C) in cultures of dissociated mouse cerebral cortex. Cultures were prepared as described in "Experimental Procedures" and subsequently incubated in media containing [U-^{13}C]glutamine (concentrations 0.1 mM, 0.3 mM and 0.5 mM) for 2.5 h as described in "Experimental Procedures". The molecular carbon labeling (MCL) was subsequently determined in cell extracts as detailed in "Experimental Procedures". Results are averages ± S.E.M. of 4–5 cultures. An asterisk indicates statistically significant differences determined by ANOVA (see "Experimental Procedures") from 0.1 mM [U-^{13}C]glutamine ($P < 0.001$) and a number sign significant differences from 0.3 mM [U-^{13}C]glutamine ($P < 0.001$)

In order to obtain information about the extent to which metabolism via the TCA cycle may be involved in synthesis of GABA from glutamine, the labeling in M + 5 (glutamate), M + 4 (GABA) representing direct synthesis without TCA cycle involvement, was compared to labeling in M + 1, M + 2 (GABA) and M + 3 (only glutamate). Figure 4 shows these results calculated as ratios. In case of

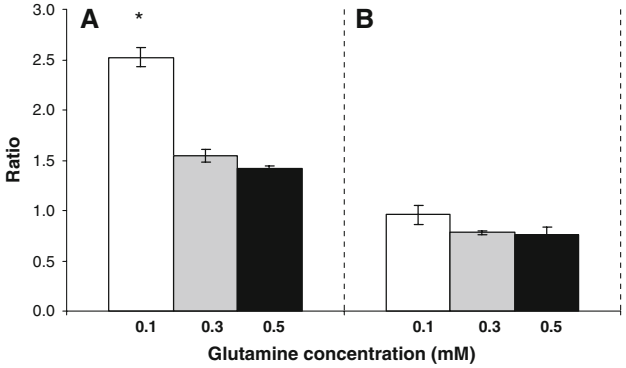

Fig. 4 Ratios of M + 5 (glutamate, A) or M + 4 (GABA, B) divided by Σ M + 3, M + 2, M + 1 and Σ M + 2, M + 1, respectively in cultures of dissociated mouse cerebral cortex. Cultures were prepared as described in "Experimental Procedures" and subsequently incubated for 2.5 h in media containing [U-^{13}C]glutamine (concentrations 0.1 mM (white bars), 0.3 mM (grey bars) and 0.5 mM (black bars)). Ratios were determined as detailed in "Experimental Procedures". Results are averages ± S.E.M. of 4–5 cultures. The asterisk indicates statistically significant differences determined by ANOVA (see "Experimental Procedures") from 0.3 mM or 0.5 mM [U-^{13}C]glutamine ($P < 0.001$)

glutamate these ratios were again dependent upon the extracellular glutamine concentration being statistically significantly higher at the low extracellular glutamine concentration. In case of GABA the ratio was about one, independent of the external glutamine concentration.

Metabolism of ^{13}C-Labeled Glucose, Lactate and Acetate

The ability of the co-cultures of neurons and astrocytes to utilize the 3 energy substrates, glucose, lactate and acetate was investigated by incubation of the cultures in a medium containing all three substrates in combination having only one of the substrates labeled with ^{13}C at any given time. This experimental paradigm allows a comparison of the three substrates to be performed regarding their metabolism via glycolysis and the TCA cycle to ultimately label the amino acids glutamine, glutamate, GABA and aspartate. The incorporation of ^{13}C (MCL) into the four amino acids from each one of the labeled substrates in the presence of the other substrates in unlabeled form is shown in Fig. 5. It is seen that the highest labeling of the amino acids was observed when lactate was the labeled substrate, followed by glucose and acetate. When acetate was the labeled substrate, glutamine had a higher incorporation of ^{13}C than glutamate ($P < 0.05$, ANOVA) whereas the opposite was found when either glucose or lactate was the labeled substrate ($P < 0.05$, ANOVA). Regardless of the labeled substrate, aspartate always was labeled to a larger extent than GABA ($P < 0.05$, ANOVA). GABA labeling was much lower when acetate was the labeled substrate compared to

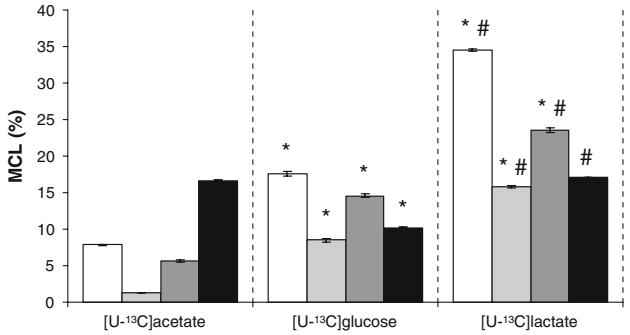

Fig. 5 Average incorporation of ^{13}C (MCL, %) in glutamate (white bars), GABA (light grey bars), aspartate (dark grey bars) or glutamine (black bars) in cultures of dissociated mouse cerebral cortex. Cultures were prepared as described in "Experimental Procedures" and subsequently incubated for 2.5 h in media containing 3 mM [U-^{13}C]acetate, 2.5 mM [U-^{13}C]glucose or 1 mM [U-^{13}C]lactate (one substrate labeled, two unlabeled as detailed in "Experimental Procedures". Molecular carbon labeling (MCL, %) was determined in cell extracts as detailed in "Experimental Procedures". Results are averages ± S.E.M. of 4–5 cultures. The asterisk indicates a significant difference from the [U-^{13}C]acetate group ($P < 0.001$; ANOVA) and a number sign a significant difference from the [U-^{13}C]glucose group ($P < 0.001$; ANOVA)

glucose and lactate, despite the high labeling of its precursors glutamine and glutamate (Fig. 5).

Since the three substrates exhibit differences with regard to the accessibility to the neuronal and astroglial compartments (see "Discussion" for references), it was of interest to analyze the relative labeling of glutamine and glutamate for each of the labeled substrates. Figure 6 shows the ratio between the total ^{13}C incorporation in glutamine and glutamate for each of the labeled substrates. It is seen that the glutamine/glutamate labeling ratio was significantly higher when acetate was the labeled substrate

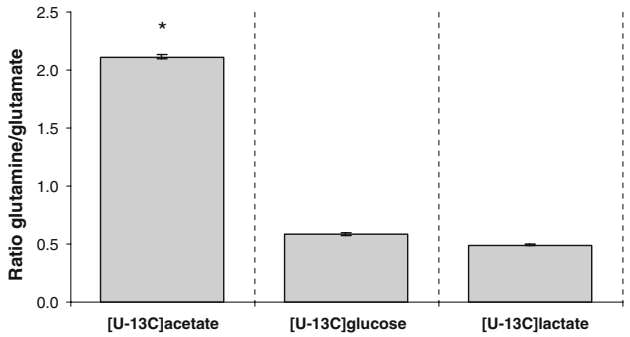

Fig. 6 Ratios of MCL in glutamate and glutamine determined in cultures of dissociated mouse cerebral cortex. Cultures were prepared as described in "Experimental Procedures" and incubated for 2.5 h in media containing 3 mM [U-^{13}C]acetate, 2.5 mM [U-^{13}C]glucose or 1 mM [U-^{13}C]lactate (one substrate labeled, two unlabeled). MCL values were determined in cell extracts as detailed in "Experimental Procedures". Results are averages ± S.E.M. of 4–5 cultures The asterisk indicates a statistically significant $P < 0.001$; ANOVA) difference from [U-^{13}C]glucose and [U-^{13}C]lactate

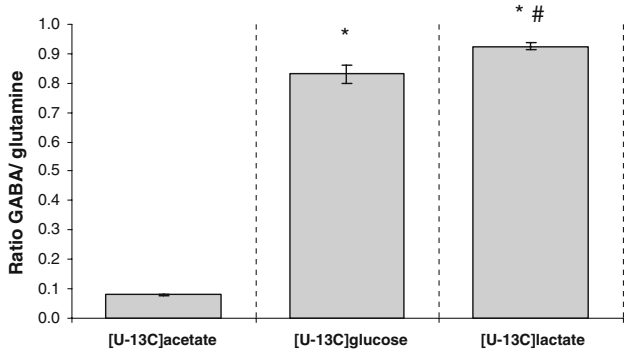

Fig. 7 Ratios of MCL in GABA and glutamine determined in cultures of dissociated mouse cerebral cortex. Cultures were prepared as described in "Experimental Procedures" and incubated for 2.5 h in media containing 3 mM [U-^{13}C]acetate, 2.5 mM [U-^{13}C]glucose or 1 mM [U-^{13}C]lactate (one substrate labeled, two unlabeled). MCL values were determined in cell extracts as detailed in "Experimental Procedures". Results are averages ± S.E.M. of 4−5 cultures. An asterisk indicates a statistically significant (ANOVA, $P < 0.001$) difference from [U-^{13}C]acetate and a number sign a difference ($P < 0.001$) from [U-^{13}C]glucose

compared to glucose or lactate being the labeled substrate. The ratio was found to be the same comparing glucose and lactate as labeled substrates. The same analysis was performed regarding the relative labeling of glutamine and GABA. Figure 7 shows that when acetate was the labeled substrate, the labeling in GABA was less than 10% of that in glutamine whereas when either glucose or lactate was the labeled substrate, the label in GABA was almost identical to that in glutamine (ratio close to 1).

Discussion

Using a co-culture system based on seeding a layer of dissociated fetal cerebral cortex cells on top of a confluent layer of cortical astrocytes it has previously been demonstrated that both glucose and acetate can function as precursors for de novo GABA synthesis, the latter substrate requiring transfer of glutamine from the astrocytic compartment [2]. The results obtained using the present much simpler co-culture system are to a large extent comparable to those obtained previously, i.e. label in glutamate and GABA was higher using glucose than acetate as the labeled substrate. Moreover, also the present culture system demonstrated a significant synthesis of glial glutamine from acetate, and subsequent transfer of glutamine from astroglia to neurons and synthesis of GABA from glutamine in the neurons. It seems therefore safe to conclude that the present much simpler co-culture system is well suited to study neuron-glia interactions with regard to GABA biosynthesis. That the content of GABA in the co-culture system to a large extent reflects the value

obtained in cultured cerebral cortical neurons [14] may also be taken as an indication that this culture system serves as a reliable model system for GABAergic neurons. The contents of glutamate and aspartate did not increase by elevating the glutamine concentration from 0.1 to 0.5 mM which is in contrast to that observed in cultured cerebral cortical neurons [14]. This may be explained by endogenous synthesis of glutamine by the astrocytes in the co-culture system. It should be noted, however, that the amino acid contents generally were higher in cultures incubated in media containing glucose, lactate and glutamine compared to those containing glucose, lactate and acetate. It thus appears that a minimum of glutamine is needed to maintain the cellular contents of glutamate, aspartate and GABA. The finding that the GABA content was decreased when the glutamine concentration in the medium was increased from 0.1 to 0.3 or 0.5 mM may be somewhat puzzling. It does, however, indicate that external glutamine and endogenous glutamine may not constitute a homogeneous pool as observed previously [15].

The concentration dependent uptake of glutamine exhibiting a tendency towards saturation and its subsequent metabolism to glutamate and GABA indicate a preferential neuronal glutamine uptake and metabolism [3, 16]. This is in agreement with the observations by Dolinska et al. [17] that cultured neural cells express the different types of glutamine transporters. Moreover, in keeping with previous findings in cerebral cortical neurons, GABA synthesis from glutamine required participation of the TCA cycle [3, 4, 16]. The extent to which GABA synthesis occurred directly from glutamine without the involvement of TCA cycle metabolism was not dependent upon the exogenous glutamine concentration, although that of glutamate decreased by elevating the glutamine concentration from 0.1 to 0.3 mM. The finding that GABA synthesis from glutamine always involved the TCA cycle to a lower extent than that for its precursor glutamate indicates that GABA may be synthesized from an inhomogeneous glutamate pool reflecting mitochondrial heterogeneity. Such compartmentation of glutamate and GABA metabolism has been demonstrated previously in mono-cultures of cerebral cortical neurons [18]. The ratio of GABA synthesis directly from glutamate compared to synthesis via the TCA cycle of approximately one (see Fig. 4B) indicates that half of the newly synthesized GABA pool originates from glutamate generated from α-ketoglutarate formed by TCA cycle metabolism of the glutamine carbon skeleton. A similar value has been found in mono-cultures of cerebral cortical neurons [18].

The best indication that the present co-culture system may be suitable as a model by which neuron-glial interactions can be investigated originates from the results concerning acetate metabolism. Acetate can only be taken up into and metabolized in astrocytes [19]. Therefore the finding in the present study that ^{13}C from [U-^{13}C]acetate was found in GABA unequivocally demonstrates transfer of its precursor glutamine from the astrocytes in the co-culture system. The extent to which this occurs reflects that of a previous study in which a different co-culture system was used [2]. It should be noted, that the MCL (%) of GABA observed after incubation with [U-^{13}C]acetate was less than 10% of that in glutamine whereas when [U-^{13}C]glutamine was the labeled substrate, GABA label was about 10–15% of that in glutamine, depending on the glutamine concentration. This may indicate that uptake of exogenous glutamine into the GABA synthesizing compartment is comparable to that from the glial compartment in which newly synthesized glutamine needs to be released prior to uptake into the GABAergic neurons. Further information about expression of the glutamine transporter mediating its release from glial cells [20] is necessary to further elucidate this issue. The very high glutamine/glutamate labeling ratio clearly shows a metabolically active glial compartment with a well functioning capacity for glutamine synthesis. This, very likely reflects a neuronal-astrocytic interaction with regard to enhancement of astrocytic glutamine synthetase activity as previously reported [21].

The labeled substrates glucose and lactate were found to act as efficient precursors for labeling in the amino acids. It was somewhat surprising that the labeling from lactate in glutamate was more pronounced than that from glucose particularly considering the fact that the co-substrate was present in unlabeled form. It should, however, be noted that cerebral cortical neurons previously have been reported to have a high capacity for lactate metabolism [14, 22, 23]. The same labeling ratios, i.e. glutamine/glutamate and GABA/glutamine, was observed using glucose and lactate as ^{13}C labeled substrate suggesting that acetyl CoA derived from these precursors is metabolized in the same cellular compartments to the same extent.

The finding that when either glucose or lactate served as the labeled substrate, the MCL ratio of glutamine to glutamate was approx. 0.5 whereas that of GABA to glutamine was about 1.0 would indicate that GABA may be synthesized from a glutamate pool originating from a heterogeneous glutamine pool. This likely reflects neuron-glial compartmentation as well as neuronal complexity with regard to GABA biosynthesis.

Overall the findings of the present study support the notion that the present simple co-culture system of GABAergic neurons and astrocytes may constitute a model system by which basic metabolic and pharmacological properties of GABAergic neurotransmission may be investigated. Currently, our laboratory is investigating the possible effects of ammonia on GABA homeostasis using this model system of neuron-glia transfer of metabolites.

Acknowledgments The expert secretarial assistance of Ms Hanne Danø and the skilful technical assistance of Ms Ann Lene Vigh are highly appreciated. The experimental work has been supported by grants from the Danish Medical Research Council (22-04-0314 and 271-07-0267) and the Lundbeck, Hørslev and Novo Nordisk Foundations. A travel grant to RL from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior—Capes is coordially acknowledged.

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Neurochem Res
DOI 10.1007/s11064-009-0062-1

ERRATUM

Erratum to: Demonstration of Neuron-Glia Transfer of Precursors for Gaba Biosynthesis in a Co-Culture System of Dissociated Mouse Cerebral Cortex

Renata Leke · Lasse K. Bak · Arne Schousboe · Helle S. Waagepetersen

Erratum to: Neurochem Res (2008) 33:2629–2635
DOI 10.1007/s11064-008-9814-6

Unfortunately Fig. 4 of this article was published wrongly. Correct version of Fig. 4 is as given below.

Also in page 2632, second column, line 3 should read 'significantly lower at the low extracellular glutamine'. Page 2634, first column, line 33 should read 'concentration, although that of glutamate increased by'.

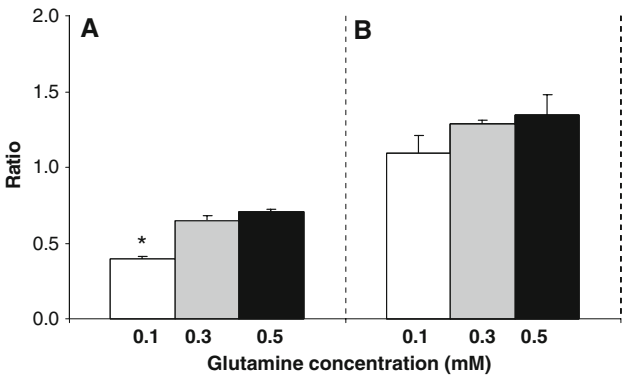

Fig. 4 Ratios of M + 5 (glutamate, A) or M + 4 (GABA, B) divided by ΣM + 3, M + 2, M + 1 and ΣM + 2, M + 1, respectively in cultures of dissociated mouse cerebral cortex. Cultures were prepared as described in ''Experimental Procedures'' and subsequently incubated for 2.5 h in media containing [U-^{13}C]glutamine (concentrations 0.1 mM (white bars), 0.3 mM (grey bars) and 0.5 mM (black bars)). Ratios were determined as detailed in ''Experimental Procedures''. Results are averages ± S.E.M. of 4–5 cultures. The asterisk indicates statistically significant differences determined by ANOVA (see ''Experimental Procedures'') from 0.3 mM or 0.5 mM U-^{13}C]glutamine ($P < 0.001$)

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CAPÍTULO II

Detoxification of ammonia in mouse cortical GABAergic cell cultures increases neuronal oxidative metabolism and reveals an emerging role for release of glucose-derived alanine

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Detoxification of ammonia in mouse cortical GABAergic cell cultures increases neuronal oxidative metabolism and reveals an emerging role for release of glucose-derived alanine

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ABSTRACT

Cerebral hyperammonemia is believed to play a pivotal role in the development of hepatic encephalopathy, a debilitating condition arising due to acute or chronic liver disease. In the brain, ammonia is thought to be detoxified *via* the activity of glutamine synthetase, an astrocytic enzyme. Moreover, it has been suggested that cerebral tricarboxylic acid (TCA) cycle metabolism is inhibited and glycolysis enhanced during hyperammonemia. The aim of the present study was to characterize the ammonia-detoxifying mechanisms as well as the effects of ammonia on energy-generating metabolic pathways in a mouse neuronal-astrocytic co-culture model of the GABAergic system. We found that 5 mM ammonium chloride affected energy metabolism by increasing the neuronal TCA cycle activity and switching the astrocytic TCA cycle towards synthesis of substrate for glutamine synthesis. In addition, ammonia exposure drastically decreased cellular ATP levels when lactate but not when glucose was the energy substrate. Furthermore, ammonia exposure enhanced the synthesis and release of alanine. Collectively, our results demonstrate that (1) neuronal oxidative metabolism is increased in the presence of ammonia, (2) synthesis and release of alanine is likely to be important for ammonia detoxification and (3) glucose but not lactate is a seminal substrate for sustaining the energy-generating pathways as well as for providing carbon skeletons for *de novo* synthesis of alanine and glutamine.

Keywords: Ammonia, Hepatic Encephalopathy, Alanine, Glutamine, Energy, Glucose, Lactate.

Abbreviations: GDH, glutamate dehydrogenase; GS, glutamine synthetase; GSH, glutathione; HE, hepatic encephalopathy; LC-MS, liquid chromatography-mass spectroscopy; MCL, molecular carbon labeling; NMRS, nuclear magnetic resonance spectroscopy; PC, pyruvate carboxylase; PDH, pyruvate dehydrogenase; TCA, tricarboxylic acid.

INTRODUCTION

Ammonia is believed to be a major etiological factor in the development of hepatic encephalopathy (HE), a neuropsychiatric syndrome that occurs as a consequence of acute or chronic liver failure (review, Hazell and Butterworth, 1999; review, Albrecht and Jones, 1999; Blei and Córdoba 2001; review, Butterworth, 2002). A high ammonia level in plasma is a characteristic feature of patients suffering from HE and a significant correlation between plasma ammonia levels and the severity of HE in acute liver failure has been demonstrated (Kramer *et al.,* 2000; review, Butterworth 2000; review, Lockwood 2004; Kundra *et al*., 2005). Likewise, the metabolic trapping of ammonia in the brain is linearly correlated to the blood ammonia levels in chronic liver patients with or without HE (Keiding *et al.*, 2006). Although the neurotoxic effects of ammonia have been extensively studied, the specific mechanisms are still not fully understood. It has previously been reported that elevated ammonia affects cellular bioenergetics, disturbs neurotransmitter systems and leads to oxidative stress (review, Butterworth, 2000; review, Rao and Norenberg, 2001). In addition, we recently showed that cerebral oxygen consumption and blood flow are both reduced in humans suffering from severe HE (Iversen *et al*., 2009). So far, focus has been on astrocytes as the major target cell for the main pathological changes associated with hyperammonemia as well as for detoxifying ammonia (review, Norenberg, 1998). The main mechanism for ammonia detoxification is believed to involve synthesis of glutamine in astrocytes (Norenberg, 1998; Buttherworth, 2002). However, a role for alanine in transfer of ammonia between brain cells has recently been proposed (Waagepetersen *et al*., 2000; Bak *et al*., 2005) and it is possible that an analogous mechanism may operate in hyperammonemia involving alanine in ammonia detoxification. We have previously investigated the effects of ammonia on cellular metabolism in a cell culture model of the glutamatergic system (Johansen *et al.*, 2007). Thus, in the present study we employed a model system of the GABAergic system, namely primary cultures of (GABAergic) cortical neurons and astrocytes. The aims of the present in vitro study were to understand (1) how GABAergic neurons and astrocytes detoxify high levels of ammonia, and (2) to explore the effect of ammonia on parameters of energy metabolism including the roles of glucose and lactate as substrates in the presence or absence of ammonia. Thus, a series of incubation experiments were performed in co-cultures of cortical neurons and astrocytes as well as cultures of cortical neurons in the presence or absence of 5 mM NH_4Cl. [1,6-

^{13}C]Glucose, [U-^{13}C]glucose, [U-^{13}C]lactate, β-[U-^{13}C]hydroxybutyrate and $^{15}NH_4Cl$ were employed as labeled precursors. After incubation, labeling of relevant intracellular amino acids was analyzed by either liquid chromatography-mass spectrometry (LC-MS) or nuclear magnetic resonance spectroscopy (NMRS). Moreover, the effect of NH_4Cl on extracellular levels of lactate and intracellular ATP levels were evaluated in both culture systems.

METHODS

Animals and chemicals

NMRI mice were obtained from Taconic M&B (Ry, Denmark). Plastic tissue culture dishes were purchased from NUNC A/S (Roskilde, Denmark), fetal bovine serum from GIBCO, Invitrogen (Taastrup, Denmark). Culture medium and poly-D-lysine (MW>300,000) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Penicillin was from Leo (Ballerup, Denmark). Lactate was measured employing the Lactic Acid Kit from Food Diagnostics (cat. no. 10 139 084 035, Grenaa, Denmark) and adenosine 5`-triphosphate (ATP) was determined employing the Bioluminescent Somatic Cell Assay Kit (Sigma Chemical Co., St. Louis, MO, U.S.A.). Isotopically labeled compounds were from either Cambridge Isotopes Laboratories, Inc. (Massachusetts, U.S.A.) or Isotec (a subsidiary of Sigma Chemical Co.). All other chemicals used were of the purest grade available from regular commercial sources.

Co-cultures of cortical neurons and astrocytes

Co-cultures of cortical neurons and astrocytes were obtained as previously described by Leke *et al*. (2008). Cells were seeded in poly-D-lysine coated 6 well plates (2 ml/well) or 80 cm^2 flasks (15 ml/flask) at a density of 2.75 x 10^6 cells/ ml and 4.0 x 10^6 cells/ml, respectively, in a slightly modified (Hertz *et al*., 1982) Dulbecco's minimum essential medium (DMEM) containing 10% (v/v) fetal calf serum. The cells were cultured for 7–8 days, a period during which they exhibited an abundant proliferation of astrocytes as well as neuronal migration as observed by light microscopy. A more detailed characterization of this culture system is provided by Leke *et al*. (2008).

Cultures of cortical neurons

Cerebral cortical neurons were isolated and cultured as described by Hertz *et al*. (1989). Cells were seeded in poly-D-lysine coated 6 well plates (2 ml/well) at a density of 2.75 x 10^6 cells/ml in a slightly modified DMEM containing 10% (v/v) fetal bovine serum. After 40–48 h in culture, 20 μM cytosine arabinoside (final concentration) was added to the culture medium to prevent astrocytic proliferation. The cells were cultured for 7–8 days.

Incubation experiments

After 7–8 days *in vitro*, cultures (either cortical neurons or co-cultures) were pre-incubated in the presence or absence (controls) of 5 mM NH_4Cl for 1h at 37°C. Subsequently, the culture medium was aspirated and the cultures were rinsed twice in phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 7.3 mM Na_2HPO_4, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, pH 7.4, 37°C). For metabolic experiments employing isotopically labeled substrates, co-cultures were subsequently incubated for 2.5h in a serum free medium containing one of the following six combinations of substrates (ii-vi were done in the presence or absence of 5 mM NH_4Cl): i) 3 mM acetate, 2.5 mM glucose, 1 mM lactate and 5 mM $^{15}NH_4Cl$ in the presence or absence of 0.5 mM glutamate (6 well plates); ii) 3 mM acetate, 2.5 mM [U-^{13}C]glucose, 1 mM lactate (6 well plates); iii) 2.5 mM [1,6-^{13}C]glucose (80 cm^2 flasks); iv) 2.5 mM [U-^{13}C]glucose (6 well plates); v) 1.0 mM [U-^{13}C]lactate (6 well plates); vi) 1.0 mM β-[U-^{13}C]hydroxybutyrate (BHB) (6 well plates). Cultured cortical neurons were incubated for 2.5h in three different incubation media in the presence or absence of 5 mM NH_4Cl: i) 2.5 mM [U-^{13}C]glucose; ii) 1.0 mM [U-^{13}C]lactate or iii) 1.0 mM [U-^{13}C]BHB. The incubation period was terminated by removing the medium from the cells which were subsequently rinsed twice with ice-cold PBS. Subsequently, the cells were extracted in 70% ethanol centrifuged (20,000 *g,* 20 min, 4°C) to separate the soluble extract from the insoluble components. The pellets were employed for protein determination. Cell extracts were lyophilized and reconstituted in water for biochemical analyses. In some instances, the incubation medium was lyophilized and reconstituted in water for quantification of extracellular amino acids and glutathione. Incubation experiments for measurement of lactate production and ATP levels were

performed in a similar manner in the presence of unlabeled substrates, as detailed in the Results section.

Biochemical analyses

^{13}C and ^{1}H NMR spectroscopy

Lyophilized cell extracts were dissolved in 200 µl 99% D_2O and pH was adjusted to values between 6.6 and 6.9. The samples were transferred into NMRS microtubes (Shigemi Inc., PA, USA). Lyophilized medium samples were dissolved in 500 µL 99% D_2O and transferred to conventional NMRS tubes. Proton decoupled ^{13}C NMRS spectra were accumulated on a BRUKER DRX600 spectrometer and the following acquisition parameters were applied: 30° pulse angle, acquisition time of 1.3 s and a relaxation delay of 0.5 s. The number of scans was typically 25,000 for each sample. Some spectra were also broad band decoupled only during acquisition to avoid nuclear Overhauser effects and accompanied by a relaxation delay of 20 s to achieve fully relaxed spectra. From several sets of spectra correction factors were obtained and applied to the integrals of the individual peaks. ^{1}H NMRS spectra were acquired with the following acquisition parameters: 90° pulse angle, an acquisition time of 1.36 s and a relaxation delay of 10 s. 64 scans were accumulated for each cell extract sample and 520 scans for each media sample. Water suppression was achieved by applying a low-power pre-saturation pulse at the water frequency. Relevant peaks in the ^{13}C and ^{1}H NMRS spectra were identified and integrated using XWINNMR software. The amounts of ^{13}C were quantified from the intervals of the peak areas and amounts of metabolites were quantified using the relevant peaks in the ^{1}H spectra. All spectra were integrated employing ethylene glycol as an internal standard and ^{1}H spectra were corrected for number of protons. NMRS data from experiments employing [1,6-^{13}C]glucose as substrate can be used for calculation of TCA cycling ratios and pyruvate carboxylase (PC)/pyruvate dehydrogenase (PDH) ratios (for further details, please see Hassel *et al.*, 1995). The first ratio is a relative measure of the activity of the TCA cycle whereas the latter ratio is a relative measure of pyruvate carboxylation (i.e. anaplerosis) vs. oxidative decarboxylation of pyruvate to acetyl-CoA for subsequent oxidation in the TCA cycle (i.e. oxidative metabolism). It should be noted that the PC/PDH ratio is an underestimation of pyruvate carboxylation due to *back cycling* of the oxaloacetate generated by the PC reaction (Merle *et al.*, 1998).

LC-MS and HPLC

The Phenomenex EZ:faast amino acid analysis kit for LC–MS was used for analysis of labeling in relevant amino acids. Mass spectrometric analysis was performed on an LC–MS system consisting of a Shimadzu LCMS-2010 mass spectrometer coupled to a Shimadzu 10A VP HPLC system. For quantification, amino acids in cell extracts and medium were separated and quantified by reversed-phase HPLC on an Agilent Eclipse AAA column (4.6x150 mm, particle size 5 μm) employing pre-column, online o-phthaldialdehyde derivatization and fluorescence detection (excitation 350 nm; detection 450 nm) as described by Geddes and Wood (1984). Data analyses for LC-MS and HPLC data were performed employing Microsoft Excel 2007 and GraphPad Prism v4.01 softwares. All labeling data were corrected for natural abundance of ^{13}C by subtracting the mass distribution of a standard containing the relevant metabolites. Isotopic enrichment was calculated according to Biemann (1962). The average percent of ^{13}C labeled carbon atoms, i.e. the percent molecular carbon labeling (MCL) was calculated as initially introduced by Bak *et al*. (2006). HPLC values were related to mg protein in cell extracts.

Assays for total protein, ATP and lactate

Protein was determined in the dissolved pellets (1 M KOH at 20^{o}C for 24 h) according to Lowry *et al*. (1951) using bovine serum albumin as the standard. To determine the amount of adenosine 5`-triphosphate (ATP) the Bioluminescent Somatic Cell Assay Kit from Sigma Chemical Co. was used and lactate production was measured employing the Lactic Acid Kit from Food Diagnostics (Grenaa, Denmark). The measurements were performed according to the manufactures' instructions on an ORION II Microplate luminometer (Berthold Detection Systems, Pforzheim, Germany). All values of ATP and lactate were corrected for protein content before they were calculated as percent of controls.

Data analysis

All data are presented as averages ± SEM and differences between groups were analyzed statistically using either one-way ANOVA followed by Bonferroni post hoc test or unpaired two-tailed Student's t-test. A *P*-value <0.05 was considered statistically significant.

RESULTS

Effect of glutamate on metabolism of NH_4Cl in co-cultures

Co-cultures of cortical mice neurons and astrocytes were incubated in the presence of 2.5 mM glucose, 1 mM lactate, 3 mM acetate and 5 mM $^{15}NH_4Cl$ in the presence or absence of 0.5 mM glutamate to investigate the effect of extracellular glutamate on metabolism of ^{15}N-labeled ammonium. Figure 1 shows the possible ^{15}N-labeling patterns in glutamate, glutamine, aspartate and alanine. Glutamine may be labeled in either the amino ([2-^{15}N]glutamine) or the amide position ([5-^{15}N]glutamine), or both. The amide position will be labeled by the reaction between glutamate and $^{15}NH_4^+$ catalyzed by glutamine synthetase (GS). Glutamine may be labeled in the amino position (and subsequently both positions) if GS acts on [2-^{15}N]glutamate formed in the reaction between α-ketoglutarate and $^{15}NH_4^+$ catalyzed by glutamate dehydrogenase (GDH). In the absence of glutamate incorporation of ^{15}N from $^{15}NH_4Cl$ into the amino or the amide groups of glutamine represented by mono-labeled glutamine was 23% (Fig. 2A), whereas double-labeled glutamine amounted to 56% (Fig. 2B). When the co-cultures were exposed to glutamate, there was a significant decrease in double-labeled glutamine to 36% (Fig. 2B) and mono-labeled glutamine showed a significant increase to 55% (Fig. 2A). In the absence of exogenous glutamate, aspartate and glutamate exhibited similar patterns of ^{15}N labeling (Fig. 2C,D). The additional presence of exogenous glutamate significantly decreased incorporation of ^{15}N into both glutamate and aspartate (Fig. 2C,D). Labeling in GABA decreased in the presence of exogenous glutamate (Fig. 2E) and alanine was labeled 37% regardless of the presence of glutamate (Fig. 2F), the latter indicating that metabolism is compartmentalized since alanine is in equilibrium with a pool of [^{15}N]glutamate that is not diluted by exogenous glutamate.

Effect of NH_4Cl on metabolism of glucose, lactate and β-hydroxybutyrate in co-cultures

Glucose is *via* a multi-step process known as glycolysis transformed to the three-carbon metabolite pyruvate. Pyruvate is in transamination equilibrium with alanine; thus, when ^{13}C-labeled glucose is the substrate, the labeling in alanine reflects the labeling in the pyruvate pool which in turn reflects glycolytic activity. The other

major fate of pyruvate is reversible reduction to lactate catalyzed by lactate dehydrogenase (LDH). This process is coupled to the NADH/NAD^+ ratio thus affecting the redox state of the cell. Co-cultures of cortical neurons and astrocytes were incubated in the presence of 2.5 mM [U-^{13}C]glucose, 1 mM lactate and 3 mM acetate in the presence or absence of 5 mM NH_4Cl to investigate the effect of NH_4Cl on glycolysis, TCA cycle metabolism and amino acid synthesis. Alanine, glutamate, glutamine, aspartate and GABA all exhibited significantly increased incorporation of ^{13}C from [U-^{13}C]glucose after exposure to NH_4Cl as evidenced by increases in MCL (%) values (Fig. 3A-E). Labeling in glutamine was increased two-fold when compared to the control condition (Fig. 3D) which indicates that its precursor glutamate was extensively labeled in the astrocytic compartment. In order to obtain more detailed information about the processes related to the TCA cycle, the effect of 5 mM NH_4Cl on the metabolism of 2.5 mM [1,6-^{13}C]glucose was investigated in co-cultures by NMRS. This technique allows us to clearly distinguish between processes taking place in neurons and astrocytes since glutamine is only synthesized in astrocytes (Norenberg and Martinez-Hernandez 1979); thus labeling in glutamine reflects labeling in the astrocytic glutamate pool whereas glutamate primarily (but not solely) reflects labeling in the neuronal glutamate pool. The TCA cycling ratios (reflecting the activity of the TCA cycle, see Methods section and Hassel *et al*., 1995) for glutamate and GABA were significantly increased when exposed to NH_4Cl (Fig. 4A) indicating that the neuronal TCA cycle activity was enhanced. On the contrary, a significant decrease in the TCA cycling ratio was observed for glutamine under similar conditions (Fig. 4A) indicating decreased TCA cycling in astrocytes. The PC/PDH ratio (see Methods section and Hassel *et al*., 1995) for glutamate synthesis increased during exposure to NH_4Cl (Fig. 4B), showing that astrocytic *de novo* synthesis *via* pyruvate carboxylation was enhanced which was also reflected by increased intracellular levels of glutamine and aspartate (Table 1). In contrast, the PC/PDH ratio for GABA was not changed when the cells were challenged with NH_4Cl (Fig. 4B). The intracellular amounts of [1,6-^{13}C]glucose and [3-^{13}C]alanine decreased and increased, respectively, whereas [3-^{13}C]lactate was unaffected by the presence of NH_4Cl (Fig. 4C). Furthermore, it was found that ammonia decreased the level of extracellular (reduced) glutathione (Table 1). In another series of experiments, metabolism of [U-^{13}C]glucose, [U-^{13}C]lactate or β-[^{13}C]hydroxybutyrate

(BHB) was evaluated in co-cultures. BHB was employed to evaluate TCA cycle metabolism since it is transformed to acetyl-CoA and metabolized in the TCA cycle independent of both glucose and lactate metabolism as well as mitochondrial and cytosolic redox states (i.e. the $NADH/NAD^+$ ratios). Thus, BHB serves merely as a tool to selectively investigate TCA cycle metabolism independent of glycolysis and metabolism of lactate. Incorporation of ^{13}C (MCL, %) into glutamate, aspartate, GABA and glutamine in co-cultures incubated in medium containing either 2.5 mM [U-^{13}C]glucose, 1 mM [U-^{13}C]lactate or 1 mM [U-^{13}C]BHB in the presence or absence of 5 mM NH_4Cl is shown in Fig. 5A-D. When glucose was the labeled substrate, all four amino acids exhibited increased incorporation of ^{13}C after exposure to NH_4Cl (Fig. 5A-D). Labeling from [U-^{13}C]lactate in glutamate, aspartate and GABA was also increased (Fig. 5A-C); however, the MCL (%) of glutamine was significantly decreased (Fig. 5D). Labeling from ^{13}C-labeled BHB in glutamate and aspartate was not affected by exposure to NH_4Cl; however, both GABA and glutamine exhibited significantly decreased incorporation of ^{13}C (Fig. 5A-D).

Effect of NH_4Cl on metabolism of glucose, lactate and β-hydroxybutyrate in cultured cortical neurons

To investigate the extent of glycolysis plus TCA cycle metabolism and amino acid synthesis in neurons challenged with NH_4Cl, cultured neurons were incubated in the presence of either 2.5 mM [U-^{13}C]glucose, 1 mM [U-^{13}C]lactate or 1 mM [U-^{13}C]BHB with or without 5 mM NH_4Cl presence (Fig. 6A-C). Incorporation of ^{13}C in glutamate and aspartate from [U-^{13}C]glucose or [U-^{13}C]lactate increased in the presence of NH_4Cl (Fig. 6A-B). GABA, on the contrary, did not exhibit a difference in ^{13}C incorporation from either [U-^{13}C]glucose or [U-^{13}C]lactate (Fig. 6C). When BHB was the labeled substrate, incorporation of ^{13}C in glutamate and GABA was increased and decreased, respectively, whereas labeling in aspartate was not affected by exposure to NH_4Cl (Fig. 6A-C).

Effect of NH_4Cl on the intra- and extracellular pools of alanine

Incorporation of label from 2.5 mM [U-^{13}C]glucose, 1 mM [U-^{13}C]lactate or 1 mM [U-^{13}C]BHB into alanine was examined in both co-cultures and cultures of cortical neurons exposed to 5 mM NH_4Cl (Fig. 7A,B). When the labeled substrate was

glucose, alanine showed an increased incorporation of ^{13}C in both culture systems after exposure to NH_4Cl (Fig. 7A-B). Furthermore, in the presence of glucose addition of NH_4Cl to co-cultures caused a significant increase in the efflux of alanine to the incubation medium (Table 1). However, when lactate was the labeled substrate, a significant decrease in ^{13}C incorporation in alanine was observed in both culture systems (Fig. 7A,B). Interestingly, it was possible to detect incorporation of ^{13}C into alanine when [U-^{13}C]BHB was employed as labeled substrate, showing that pyruvate re-cycling was active in both culture systems and that it increased in cultured neurons in the presence of NH_4Cl (Fig. 7B). Since a potential role for alanine in detoxification of ammonia emerged from the present study the results regarding this aspect from the individual experiments are presented in here (Fig. 7) for comparison.

Effect of NH_4Cl on ATP levels in co-cultures and cultures of neurons

The ATP levels were determined in co-cultures and cultures of cortical neurons exposed to 5 mM NH_4Cl for a total of 3.5h (as in the labeling experiments) to evaluate the impact of glucose (2.5 mM), lactate (1 mM) or BHB (1 mM) as substrates on the energy charge of the cells. The level of ATP in the presence of glucose without exposure to NH_4Cl was set to 100% and all other conditions were related to this value. When glucose was the substrate, a small decrease in the ATP level was observed in neurons but not in co-cultures after exposure to NH_4Cl (Fig. 8A,B). Lactate, however, was not able to maintain the levels of ATP in neither co-cultures nor cultured neurons when challenged with NH_4Cl (Fig. 8A,B). When cultured neurons were supplied with both glucose and lactate as substrates, the level of ATP was lower than when each substrate was present alone (Fig. 8B). Employing BHB as the substrate, the level of ATP was lower for both culture systems when compared to the levels in the presence of glucose or lactate and BHB was not able to maintain the ATP levels after treatment with NH_4Cl.

Effect of NH_4Cl on release of lactate from co-cultures and cultures of neurons

Extracellular levels of lactate were measured in the incubation medium of both co-cultures and cultures of cortical neurons after incubation for 3.5 h in the presence of 2.5 mM glucose as energy substrate with or without 5 mM NH_4Cl. Values in the presence of NH_4Cl were normalized to the control condition (i.e absence of NH_4Cl).

Incubation with NH_4Cl led to an increase in the amount of lactate in the extracellular compartment of about 208% and 147% in co-cultures and cultures of cortical neurons, respectively (Fig. 9).

DISCUSSION

It is well established that ammonia directly or indirectly affects different cellular processes such as neuronal and astrocytic energy metabolism as well as neurotransmitter systems function, which will lead to some of the deleterious effects observed in HE (review, Albrecht and Jones, 1999). We have previously investigated the metabolic pathways for detoxification of ammonia as well as the consequences for energy metabolism of high levels of ammonia (2 and 5 mM) in cultured cerebellar (glutamatergic) neurons and astrocytes as well as in a rat model of chronic HE (Johansen *et al*., 2007; Bak *et al*., 2009). Furthermore, we recently investigated cerebral oxygen consumption and blood flow by positron emission tomography during an acute episode of HE in patients suffering from liver cirrhosis (Iversen *et al*., 2009). Here, we expand on these studies to include a novel cortical (GABAergic) neuronal-astrocytic co-culture system (Leke *et al*., 2008) to specifically investigate the effects of ammonia on the GABAergic system. Several studies have implicated that the GABAergic system is affected by ammonia toxicity (e.g. Basile 2002; Jones 2003; Ahboucha & Butterworht 2004; 2008; Cauli *et al*., 2009); thus, the present study focuses on uncovering the cellular mechanisms affected during an acute load of ammonia. The co-culture system was chosen over mono-cultures in order to investigate the effects of high ammonia levels in a more intact system that more closely resembles the *in vivo* situation. The level of ammonia employed in this study (5 mM) is probably well above what the cells would ever experience *in vivo*; however, the aim of this study was to investigate the acute cellular effects of a high ammonia load and *in vitro* cell culture-based studies on acute ammonia toxicity is routinely done employing millimolar levels of ammonia.

Detoxification of ammonia: role of alanine

Ammonia detoxification is believed to occur mainly by synthesis of glutamine *via* GS activity (Cooper and Plum, 1987) which is selectively expressed in astrocytes (Norenberg and Martinez-Hernandez, 1979). However, it has been demonstrated that

GS has little spare capacity to adapt to hyperammonemia (Cooper *et al*., 1985; Bosman *et al*, 1990; Kanamori *et al*.1996; Zwingmann *et al*, 2003). Glutamate synthesis from α-ketoglutarate *via* GDH is a pathway for ammonia fixation in both neurons and astrocytes (Fig. 1; Yudkoff *et al*., 1990; Ott *et al*. 2005; review, Schousboe and Waagepetersen, 2007). However, since neurons lack PC activity they are unable to perform *de novo* synthesis of α-ketoglutarate and are thus not capable of employing this pathway for detoxification of ammonia (review, Hertz *et al*., 1999). In the present experiments, labeling of both aspartate and alanine in co-cultures from $^{15}NH_4Cl$ was close to 40% suggesting that transamination reactions following reductive amination by GDH is an important pathway for ammonia detoxification in these cultures. Most likely, alanine is in equilibrium with a small pool of glutamate that is continuously being synthesized by GDH thus detoxifying ammonia (Figs. 1 and 10); this process would be analogous to the lactate-alanine shuttle system suggested to operate at the glutamatergic synapse (Waagepetersen *et al*., 2000; Zwingmann *et al*., 2000; Bak *et al*., 2005). Interestingly, synthesis and elevated levels of alanine have been demonstrated in different studies employing both brain cell cultures and rodent models of hyperammonemia (Hindfelt *et al*., 1977; Zwingmann *et al*., 2003; Chatauret *et al*., 2003; Zwingmann and Leibfritz 2005; Johansen *et al*., 2007). Since the blood-brain barrier has transport systems for alanine (review, Hawkins *et al*., 2006), one possibility is that synthesis and subsequent release of alanine to the extracellular space and further on into the blood stream constitutes a detoxification mechanism distinct from astrocytic synthesis and export of glutamine (Fig. 10). In support of this, increased brain and blood levels of alanine were found in an animal model of acute liver failure at pre-coma and coma stages (Zwingmann *et al*., 2003). Here, we observed an increased synthesis of [3-^{13}C]alanine from [1,6-^{13}C]glucose in co-cultures in the presence of NH_4Cl. This was accompanied by an extracellular accumulation of alanine, suggesting that alanine was synthesized and subsequently released from the cells. In agreement with this, Chatauret *et al*. (2003) demonstrated that *de novo* synthesis of alanine from glucose increased according to the degree of encephalopathy and brain edema in a rat model of acute liver failure. It is likely that both *de novo* alanine synthesis and subsequent release are occurring in both astrocytes and neurons (Fig. 10); however, it should be underlined that such a mechanism is the only way for neurons to detoxify ammonia. Interestingly, since

labeling in alanine from [U-^{13}C]lactate was decreased in the presence of ammonia whereas that from [U-^{13}C]glucose was increased in both culture systems (Fig. 7), lactate seemed to be less efficient in supporting formation of alanine compared to glucose. Thus, glucose as substrate is seminal for *de novo* synthesis of alanine in the presence of high levels of ammonia. However, the importance of neuronal synthesis of alanine as a putative mean of detoxifying ammonia remains speculative at this point awaiting eventual corroborating experimental evidence.

Detoxification of ammonia: role of glutamate and glutamine

In addition to the novel aspect of alanine being important for GABAergic neuronal detoxification of ammonia, we found that co-cultures incubated with $^{15}NH_4Cl$ exhibited high double-labeling of glutamine indicating that both GS and GDH were highly active in astrocytic ammonia fixation (Fig. 1). Addition of unlabeled glutamate to the co-cultures incubated in the presence of $^{15}NH_4Cl$ diminished the relative amount of double-labeled glutamine whereas the relative amount of mono-labeled glutamine (presumably labeled in the amide group) was noticeably increased. This effect was probably due to dilution from unlabeled glutamate. Since glutamine is only formed in astrocytes (Norenberg and Martinez-Hernandez, 1979), this clearly shows that when ample exogenous glutamate is present the main detoxifying pathway in astrocytes is amidation of glutamate to glutamine which might subsequently leave the brain (review, Hawkins *et al*., 2006). It has been demonstrated that physiological concentrations of [^{13}N]ammonia infused *via* the internal carotid artery in conscious rats resulted in the specific activity of glutamine being four hundred times that of glutamate (Cooper *et al*., 1979), indicating that ammonia is mainly metabolized *via* a small glutamate pool with a high turnover being distinct from the large glutamate pool (Berl *et al*., 1962; Cooper *et al*., 1979; review, Cooper., 2001). The enzyme pyruvate carboxylase (PC) is necessary for *de novo* synthesis of α-ketoglutarate, the precursor for glutamate and thus glutamine. The activity of PC is known to be specific to astrocytes and it is the most important enzyme for anaplerosis in the brain (Patel, 1974; Yu *et al*., 1983; Shank *et al*., 1985; Cesar and Hamprecht, 1995; Waagepetersen *et al*., 2002; Lovatt *et al*., 2007). Here, co-cultures exhibited a significant increase in the PC/pyruvate dehydrogenase (PDH) ratio for glutamate, showing that during a period of ammonia-induced toxicity the flux of carbon through the PC reaction was increased relative to oxidation in the TCA cycle. In accordance with this, the total

intracellular amount of glutamine was doubled in co-cultures exposed to NH_4Cl. Previous studies found increased anaplerosis both in cultured astrocytes incubated in the presence of ammonia and in animal models of hyperammonemia (Lapidot and Gopher, 1997; Zwingmann *et al*., 1998; Kanamatsu and Tsukada, 1999; Zwingmann *et al*., 2003 review, Zwingmann and Butterworth, 2005; review, Zwingmann, 2007). Thus, anaplerotic flux through the PC reaction is coupled to nitrogen removal and that is confirmed in the present study employing a GABAergic system. It is well described that the levels of glutamine are increased during hyperammonemia (Zwingmann and Leibfritz, 2005). Moreover, a high level of this amino acid has been related to disturbances of the glutamate-glutamine cycle, free radical production and oxidative stress (Jayakumar *et al*., 2004; Rama Rao 2005). In addition, glutamine has been related to astrocyte swelling and brain edema by acting as an osmolyte drawing water into the cells (Brusilow and Traystman 1986; Swain *et al*., 1992; review, Norenberg *et al*., 2005). Although recent studies have questioned the role of glutamine accumulation for the development of brain edema (Chatauret *et al*., 2001; Zwingman *et al*., 2003; Zwingman, 2007), it is believed that a raised glutamine level is involved in free radial production and the induction of the mitochondrial permeability transition (Rama Rao *et al*., 2003; Jayakumar *et al*., 2004;2006). Interestingly, the concentration of the endogenous antioxidant glutathione (reduced form) was decreased in the extracellular compartment of co-cultures after incubation with NH_4Cl indicating that glutathione is being oxidized and/or that glutamate is being redirected to synthesis of glutamine rather than glutathione (cf. above and Kosenko *et al*., 1997; Murthy *et al*., 2001, Rama Rao *et al*., 2003).

Effect of ammonia on energy-generating pathways: role of glucose and lactate as substrates

An early study from Bessman and Bessman (1955) suggested that the (astrocytic) TCA cycle was deprived of α-ketoglutarate during hyperammonemia due to its utilization for glutamine synthesis. Moreover, ammonia has been suggested to inhibit both α-ketoglutarate dehydrogenase (Lai and Cooper, 1986) and PDH, the latter resulting in a decrease in TCA cycle activity and an increase in lactate production (Zwingmann *et al*., 2003). Increased glycolysis and concomitant lactate production during hyperammonemia has also been suggested to be a consequence of increased

cytosolic levels of NADH due to inhibition of the malate-aspartate shuttle caused by a lowered amount of cytosolic glutamate (Hindfelt *et al*., 1977; Kala and Hertz, 2005). In the present study, we observed increased incorporation of ^{13}C from [U-^{13}C]glucose into glutamate and aspartate in both culture systems as a consequence of treatment with NH_4Cl. These results indicate that the neuronal TCA cycling was increased by ammonia. In agreement with this, the NMRS data based on metabolism of [1,6-^{13}C]glucose in co-cultures revealed that the neuronal TCA cycling was increased while the astrocytic TCA cycling was reduced in the presence of NH_4Cl. The reduction in the astrocytic TCA cycle rate is likely due to withdrawal of α-ketoglutarate from the TCA cycle for synthesis of glutamate and glutamine. Labeling in glutamate, aspartate and GABA from ^{13}C-labeled lactate increased in the co-cultures treated with NH_4Cl while labeling in glutamine decreased. This is probably because lactate as the sole substrate is not sufficient to sustain the cells both energetically and supply them with carbon for *de novo* synthesis of glutamine. In addition, labeling from [U-^{13}C]lactate into glutamate and aspartate was also increased in neuronal cultures indicating an increase in TCA cycle activity. To further study the effect of ammonia toxicity on oxidative metabolism, we have employed β-[U-^{13}C]hydroxybutyrate (BHB) as a substrate to evaluate TCA cycle metabolism independent of both glucose and lactate metabolism as well as cytosolic NADH/NAD^+. In co-cultures, incorporation of ^{13}C from [U-^{13}C]BHB did not increase in neither glutamate nor aspartate and labeling in GABA and glutamine was diminished during ammonia-induced toxicity. However, cortical neurons presented an increase in ^{13}C incorporation into glutamate under the same conditions, indicating increased cycling in the TCA cycle thus confirming the observed increase in neuronal oxidative metabolism when ^{13}C-labeled glucose and lactate were substrates. It should be noted, that when BHB was supplied as the only substrate neither co-cultures nor cultured neurons were able to maintain ATP levels compared to the situation when glucose and lactate were substrates. A number of studies have demonstrated that glycolysis is enhanced during ammonia toxicity (e.g. Ratnakumari and Murthy 1992;1993; Johansen *et al*., 2007). In the present work, we observed ammonia-induced increases in the amount of [3-^{13}C]alanine from [1,6-^{13}C]glucose as well as increases in MCL (%) value of alanine from metabolism of [U-^{13}C]glucose in co-cultures and cultured neurons, respectively. Thus, glycolysis is enhanced in both

cortical neurons and astrocytes in the presence of NH_4Cl. In strong support of this, both cultured neurons and co-cultures released high amounts of lactate in the presence of NH_4Cl. Elevated levels of lactate have been described previously and suggested to contribute to the pathogenesis of HE since it is related to astrocyte swelling and brain edema (Zwingmann *et al*., 2003; Tofteng and Larsen, 2002; Chatauret *et al*., 2003). In contrast, a study by Kala and Hertz (2005) found no effect on lactate production in cultures of cortical and cerebellar granule neurons. In the present work, co-cultures responded with a relatively larger increase in extracellular lactate compared to cultured neurons. This indicates that cortical astrocytes increase anaerobic glycolysis considerably which is in contrast to cultured cerebellar astrocytes that only showed a marginal increase in glycolysis (Johansen *et al*., 2007). Interestingly, the ATP level in both culture systems fell markedly after exposure to NH_4Cl when lactate but not when glucose was the substrate. This clearly indicates that lactate is not able to support energy production under these conditions; even in the presence of both substrates, the ATP level was negatively affected by NH_4Cl. This is likely due to the fact that when exogenous lactate is metabolized to pyruvate by LDH, the $NADH/NAD^+$ increases thus inhibiting glycolysis; this is especially detrimental if the malate-aspartate shuttle is inhibited, as has been suggested (Hindfelt *et al*., 1977; Hertz and Kala, 2007). Hindfelt *et al*. (1977) demonstrated a decrease in cerebral ATP level in portacaval-shunted rats infused with ammonia which has also been observed in cultured astrocytes treated with NH_4Cl (Haghigaht and McCandless, 1997). More recently, a study performed by Zwingmann and Leibfritz (2005) measured the high energy phosphates by ^{31}P NMRS and demonstrated that cultured astrocytes incubated with 5 mM glucose and 5 mM NH_4Cl decreased the ATP level to 72% of the control value. We recently showed that both cerebral oxygen consumption and blood flow are reduced during an acute episode of HE in patients suffering from liver cirrhosis (Iversen *et al*., 2009); thus, in the intact brain the net effect of acute HE/hyperammonemia is a reduction in either supply or need of oxygen. How this ties in with the present and earlier findings of increased oxidative metabolism in glutamatergic (Johansen *et al*., 2007) and GABAergic neurons (present work) remains unclear; however, a massive increase in anaerobic glycolysis leading to lactate production and release might result in decreased oxygen consumption even in the face of increased neuronal oxidative metabolism.

CONCLUSION

Here, we provide evidence implicating *de novo* synthesis of alanine as a novel and potentially important mechanism for disposal of ammonia for the GABAergic system; it is suggested that *de novo* synthesis of alanine is crucial for neuronal detoxification of ammonia. Furthermore, we provide evidence that ammonia up-regulates glycolysis in both GABAergic neurons and astrocytes, increases neuronal oxidative metabolism and switches the astrocytic TCA cycle towards anaplerosis rather than oxidation. Finally, we found that glucose rather than lactate as a substrate is seminal for maintaining the cellular energy charge in the form of ATP in the presence of ammonia. Collectively, this suggests that GABAergic neurons respond to an acute high load of ammonia with an increase in glycolysis to produce ATP, to fuel the TCA cycle and to provide substrate for alanine synthesis; on the other hand, astrocytes increase glycolysis to produce ATP and substrate for anaplerotic reactions for *de novo* synthesis of glutamate, glutamine and alanine.

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Table 1

Quantification of amino acids and glutathione (nmol/mg protein) in incubation medium and cell extracts of cortical co-cultures after incubation in the presence or absence of 5 mM NH_4Cl

Analyte	Condition	Cell extracts (nmol/mg protein)	Incubation medium (nmol/mg protein)
Glutamine	Control	6.6 ± 0.7	145.8 ± 8.0
	NH_4Cl	13.3 ± 0.7*	214.5 ± 31.7
Glutamate	Control	54.7 ± 3.0	n.d.
	NH_4Cl	61.1 ± 4.8	n.d.
Aspartate	Control	27.7 ± 1.2	2.7 ± 0.5
	NH_4Cl	20.8 ± 1.4*	2.5 ± 0.5
GABA	Control	14.5 ± 1.0	n.d.
	NH_4Cl	13.5 ± 0.4	n.d.
Alanine	Control	35.4 ± 4.2	31.7 ± 1.7
	NH_4Cl	26.4 ± 2.4	36.3 ± 0.4*
Glutathione	Control	n.d.	4.0 ± 0.1
	NH_4Cl	n.d.	3.4 ± 0.1*

Co–cultures were incubated in the presence of 2.5 mM glucose in the presence (NH_4Cl) or absence (control) of 5 mM NH_4Cl (see Methods section). Intracellular and extracellular amounts (nmol/mg protein) of glutamine, glutamate, aspartate, GABA, alanine and glutathione were measured by HPLC. Results are averages ± SEM of 4 cultures. *, significantly different from the control condition ($P < 0.05$). n.d., not detectable.

Legends

Fig. 1

The possible patterns of ^{15}N-labeling in glutamate, glutamine, aspartate and alanine. AAT, aspartate aminotransferase; ALAT, alanine aminotransferase; GDH, glutamate dehydrogenase; GS, glutamine synthetase (astrocytes only).

Fig. 2

Relative quantification of isotopic labeling in intracellular amino acids in cortical co-cultures following incubation in the presence of $^{15}NH_4Cl$. Co-cultures were incubated in the presence of 5 mM [^{15}N]NH_4Cl, 3 mM acetate, 2.5 mM glucose and 1 mM lactate in the presence (black bars) or absence (gray bars) of 0.5 mM of glutamate (see Methods section). Incorporation of ^{15}N into glutamine (A,B; mono- and double-labeled, respectively), glutamate (C), aspartate (D), GABA (E) and alanine (F) were measured by LC-MS. Results are averages ± SEM of 6 cultures. *, significantly different from the control condition (P<0.05).

Fig. 3

Relative quantification of isotopic labeling in intracellular amino acids in cortical co-cultures following incubation in the presence of [U-^{13}C]glucose. Co-cultures were incubated in the presence of 2.5 mM [U-^{13}C]glucose, 1 mM lactate and 3 mM acetate in the presence (black bars) or absence (gray bars) of 5 mM NH_4Cl (see Methods section). Incorporation of ^{13}C expressed as MCL (%; see Methods section) into glutamate (A), aspartate (B), GABA (C), glutamine (D) and alanine (E) were measured by LC-MS. *, significantly different from the control condition (P<0.05).

Fig. 4

Absolute quantification of isotopic labeling in intracellular glucose, alanine and lactate as well as TCA cycling and PC/PDH ratios in cortical co-cultures following incubation in the presence of [1,6-^{13}C]glucose. Co–cultures were incubated in the presence of 2.5 mM [1,6-^{13}C]glucose in the presence (black bars) or absence (gray bars) of 5 mM NH_4Cl (see Methods section). TCA cycling ratios for glutamate (glu), glutamine (gln) and GABA (A) as well as PC/PDH ratios for glutamate and GABA (B) were calculated based on NMRS data (see Methods section). Likewise,

intracellular amounts (nmol/mg protein) of [1-^{13}C]glucose (Glc), [3-^{13}C]alanine (Ala C-3) and [3-^{13}C]lactate (Lac C-3) were determined from NMRS data by employing an internal standard (C; see Methods section). Results are averages ± SEM of 4 cultures. *, significantly different from the control condition (P<0.05).

Fig. 5

Relative quantification of isotopic labeling in intracellular amino acids in cortical co-cultures following incubation in the presence of uniformly ^{13}C-labeled glucose, lactate or BHB. Co–cultures were incubated in the presence of either 2.5 mM [U-^{13}C]glucose (Glc), 1.0 mM [U-^{13}C]lactate (Lac) or 1.0 mM [U-^{13}C]BHB (BHB) in the presence (black bars) or absence (gray bars) of 5 mM NH_4Cl (see Methods section). Incorporation of ^{13}C expressed as MCL (%; see Methods section) into glutamate (A), aspartate (B), GABA (C) and glutamine (D) were measured by LC-MS. Results are averages ± SEM of 3 cultures. *, significantly different from the control condition (P<0.05).

Fig. 6

Relative quantification of isotopic labeling in intracellular amino acids in cultured cortical neurons following incubation in the presence of uniformly ^{13}C-labeled glucose, lactate or BHB. Cultured cortical neurons were incubated in the presence of either 2.5 mM [U-^{13}C]glucose (Glc), 1.0 mM [U-^{13}C]lactate (Lac) or 1.0 mM [U-^{13}C]BHB (BHB) in the presence (black bars) or absence (gray bars) of 5 mM NH_4Cl (see Methods section). Incorporation of ^{13}C expressed as MCL (%; see Methods section) into glutamate (A), aspartate (B) and GABA (C) were measured by LC-MS. Results are averages ± SEM of 3 cultures. *, significantly different from the control condition (P<0.05).

Fig. 7

Relative quantification of isotopic labeling in intracellular alanine in co-cultures or cultured cortical neurons following incubation in the presence of uniformly ^{13}C-labeled glucose, lactate or BHB. Cortical co-cultures (A) or cultured cortical neurons (B) were incubated in the presence of 2.5 mM [U-^{13}C]glucose (Glc), 1.0 mM [U-^{13}C]lactate (Lac) or 1.0 mM [U-^{13}C]BHB (BHB) in the presence (black bars) or absence (gray bars) of 5 mM NH_4Cl (see Methods section). Incorporation of ^{13}C

expressed as MCL (%; see Methods section) into alanine were measured by LC-MS. Results are averages ± SEM of 3 cultures. *, significantly different from the control condition (P<0.05).

Fig. 8

Relative quantification of ATP levels in co-cultures or cultured cortical neurons following incubation in the presence of glucose, lactate or BHB. Cortical co-cultures (A) or cultured cortical neurons (B) were incubated in the presence of 2.5 mM glucose (Glc), 1.0 mM lactate (Lac), 1.0 mM BHB (BHB) or the combination of glucose and lactate (neurons only) in the presence (black bars) or absence (gray bars) of 5 mM NH_4Cl (see Methods section). Levels of ATP were measured enzymatically and expressed as percent relative to the absolute level in the presence of glucose. Results are averages ± SEM of 3 to 12 cultures. *, significantly different from the individual control conditions (P<0.05); §, significantly different from the glucose control condition (P<0.05); #, significantly different from the lactate control condition (P<0.05).

Fig. 9

Relative quantification of extracellular levels of lactate in co-cultures or cultured cortical neurons following incubation in the presence of uniformly ^{13}C-labeled glucose, lactate or BHB. Cortical co-cultures or cultured cortical neurons were incubated in the presence of 2.5 mM glucose in the presence (black bars) or absence (gray bars) of 5 mM NH_4Cl (see Methods section). Levels of lactate were measured enzymatically and expressed as percent relative to the control condition (i.e. absence of NH_4Cl). Results are averages ± SEM of 5-7 cultures. *, significantly different from the control condition (P<0.05).

Fig. 10

Schematic cartoon depicting the mechanisms involved in fixation of excess ammonia into glutamine and alanine. We hypothesize that release of glucose-derived alanine into the bloodstream might constitute a novel and quantitatively important pathway for ammonia detoxification, supplementing the known pathway of glutamine synthesis and release. See Discussion for further details. aKG, α-ketoglutarate; Ala,

alanine; ALAT, alanine aminotransferase; GDH, glutamate dehydrogenase; Glc, glucose; Glu, glutamate; Gln, glutamine; Lac, lactate; GS, glutamine synthetase; LDH, lactate dehydrogenase; OAA, oxaloacetic acid; PC, pyruvate carboxylase; Pyr, pyruvate; TCA, tricarboxylic acid (cycle).

Fig. 1

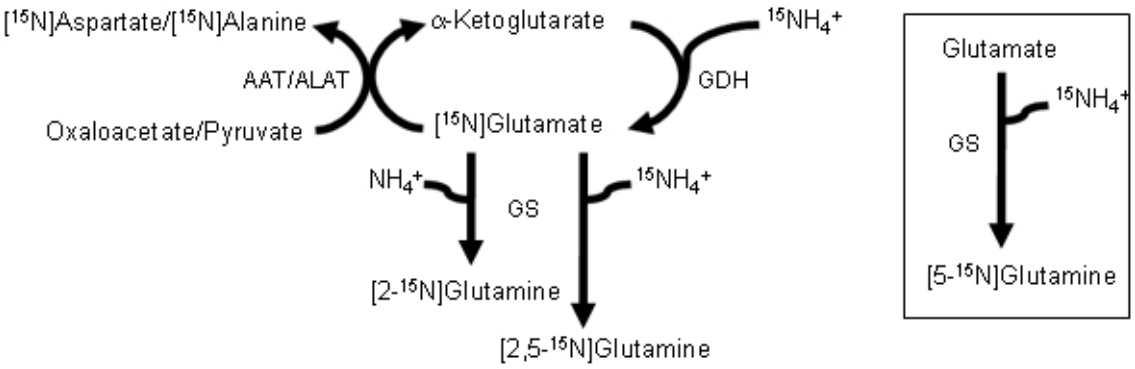

[15N]Aspartate/[15N]Alanine
α-Ketoglutarate
15NH4+
AAT/ALAT
GDH
Oxaloacetate/Pyruvate
[15N]Glutamate
NH4+
GS
15NH4+
[2-15N]Glutamine
[2,5-15N]Glutamine
Glutamate
15NH4+
GS
[5-15N]Glutamine

Fig. 2

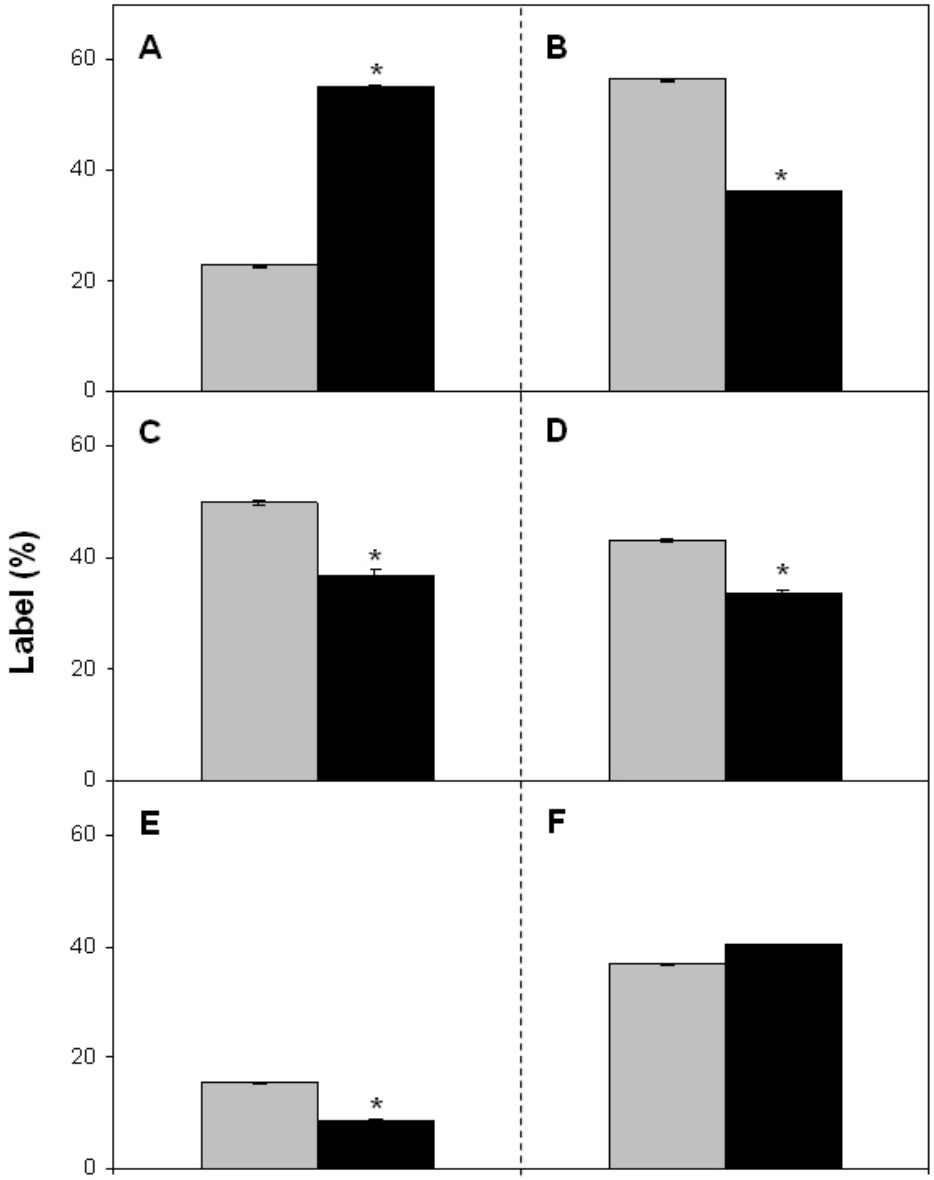


Fig. 3

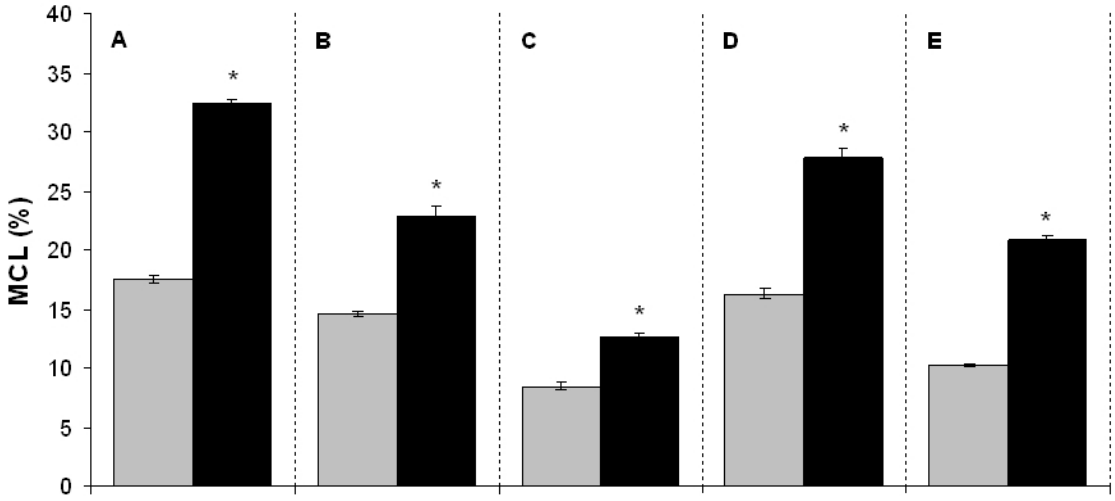


Fig. 4

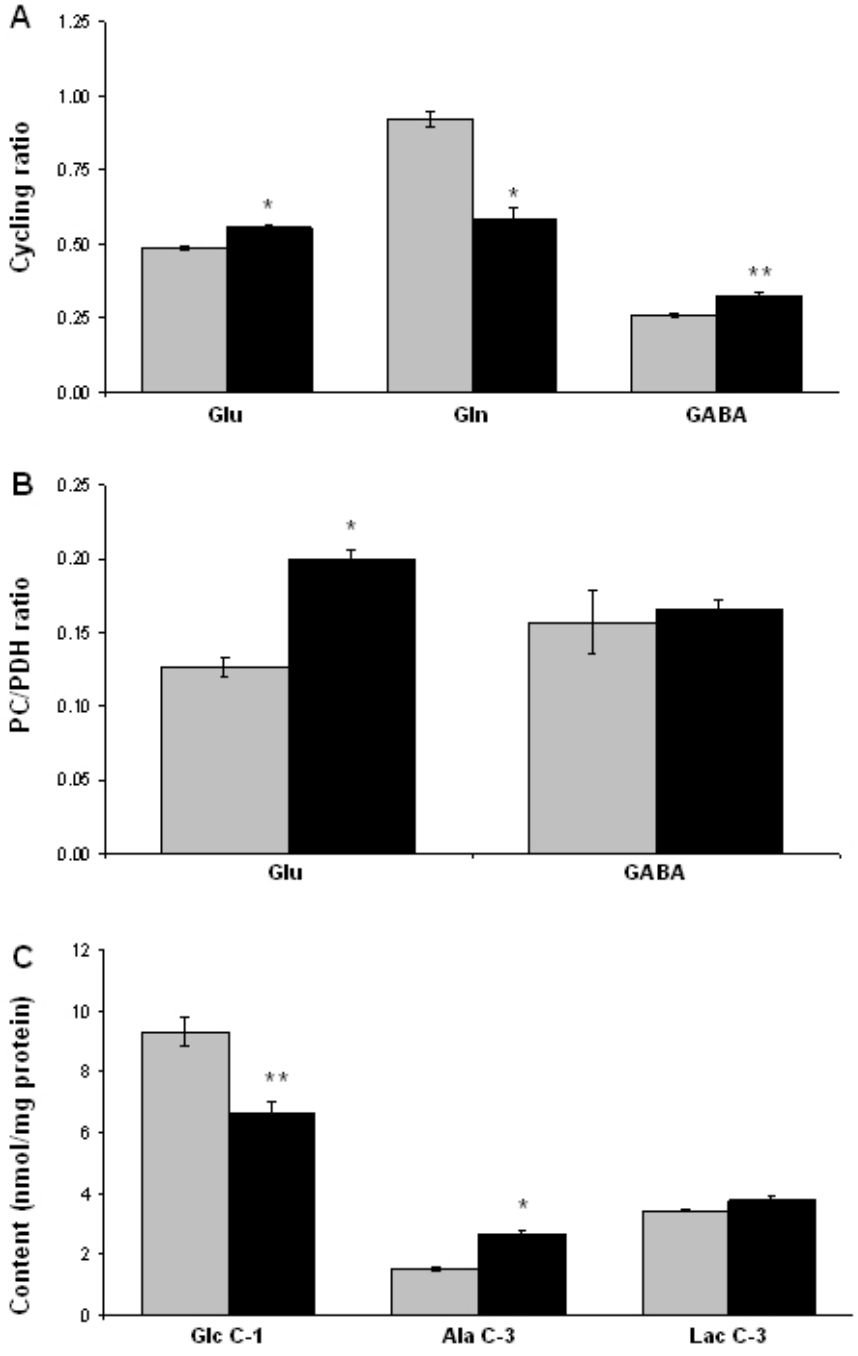

A
Cycling ratio
1.25
1.00
0.75
0.50
0.25
0.00
*
*
**
Glu
Gln
GABA
B
PC/PDH ratio
0.25
0.20
0.15
0.10
0.05
0.00
*
Glu
GABA
C
Content (nmol/mg protein)
12
10
8
6
4
2
0
**
*
Glc C-1
Ala C-3
Lac C-3

Fig. 5

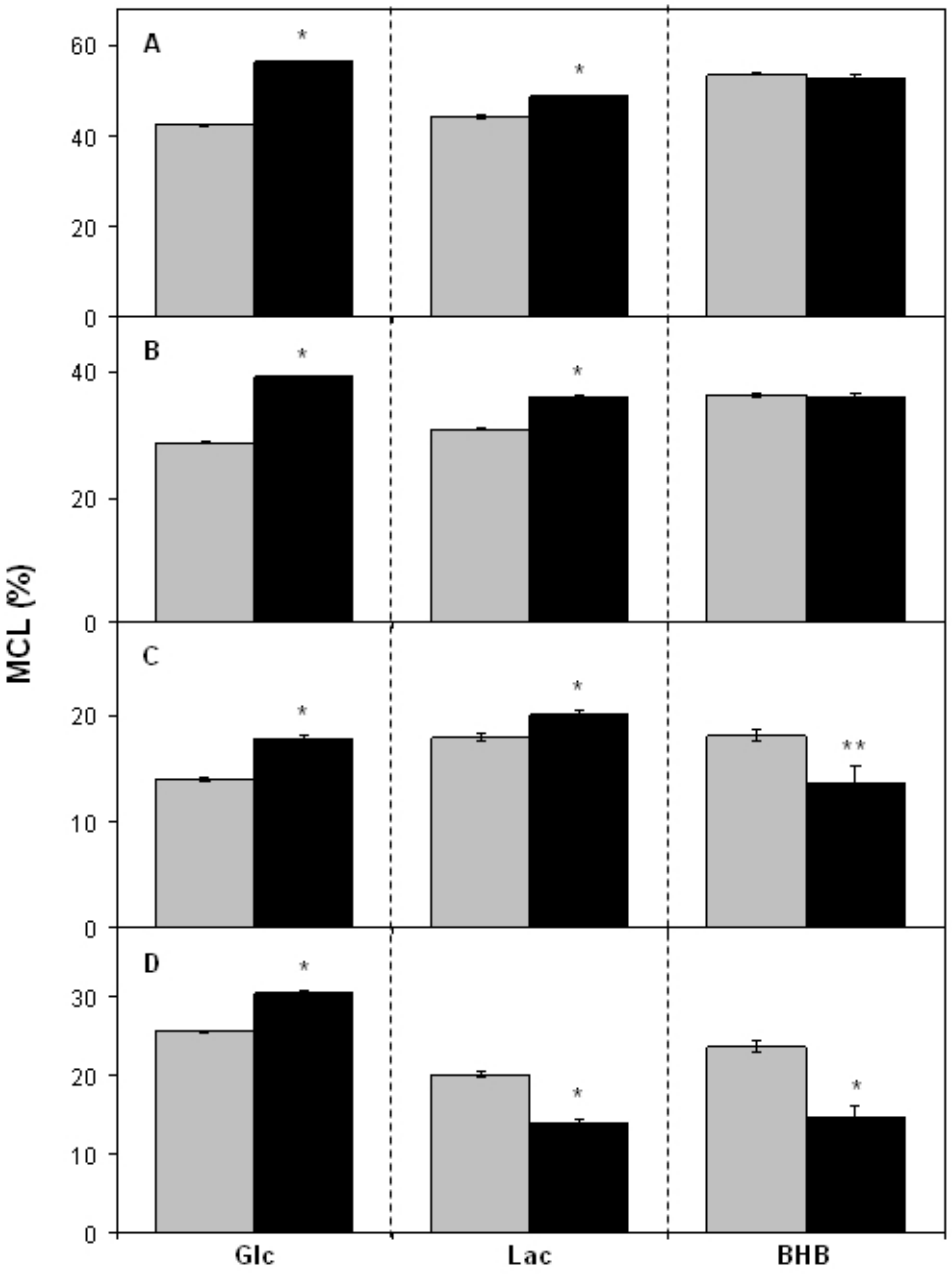


Fig. 6

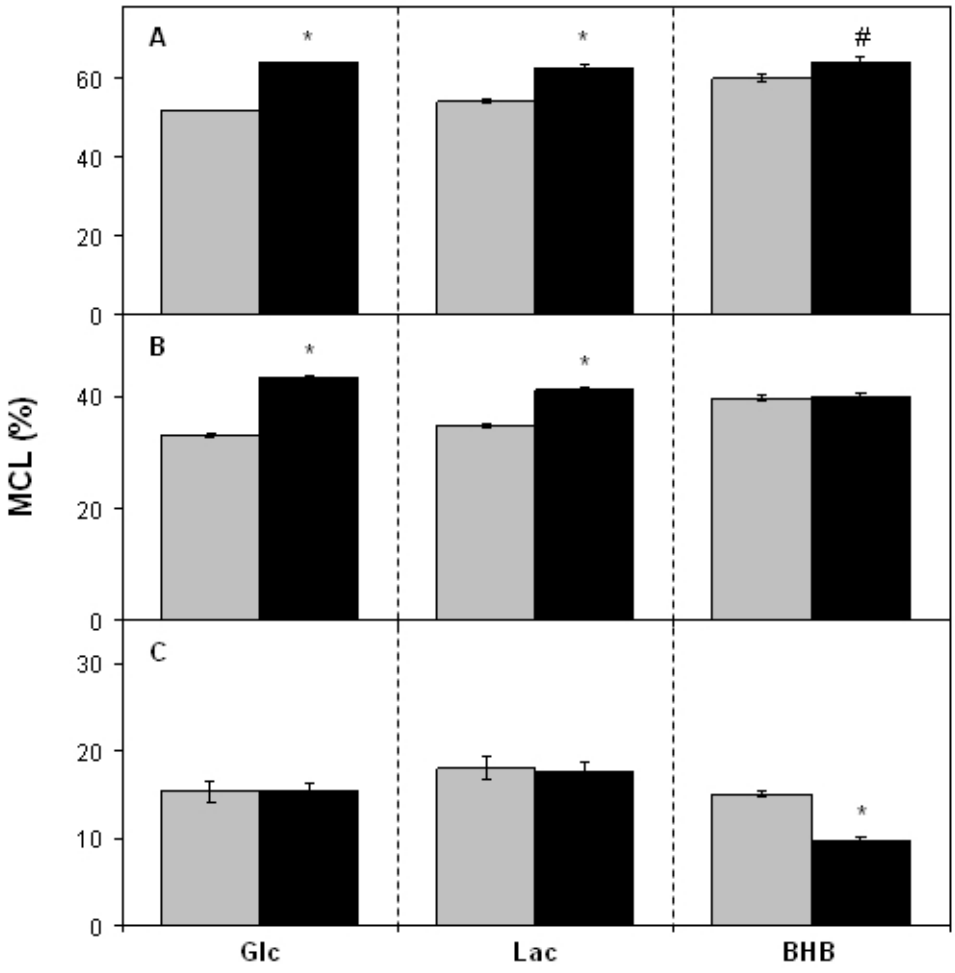


Fig. 7

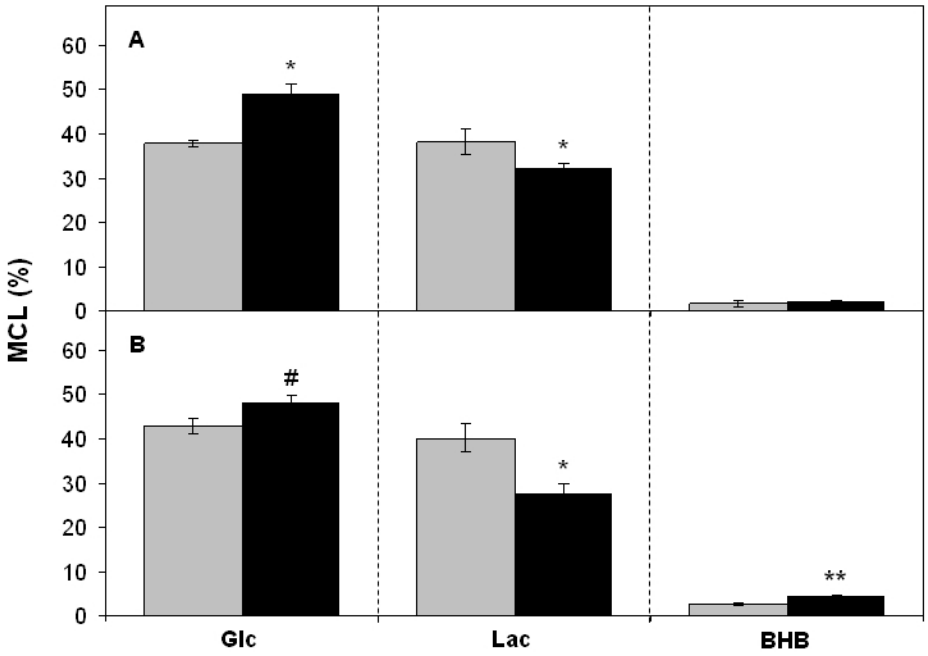


Fig. 8

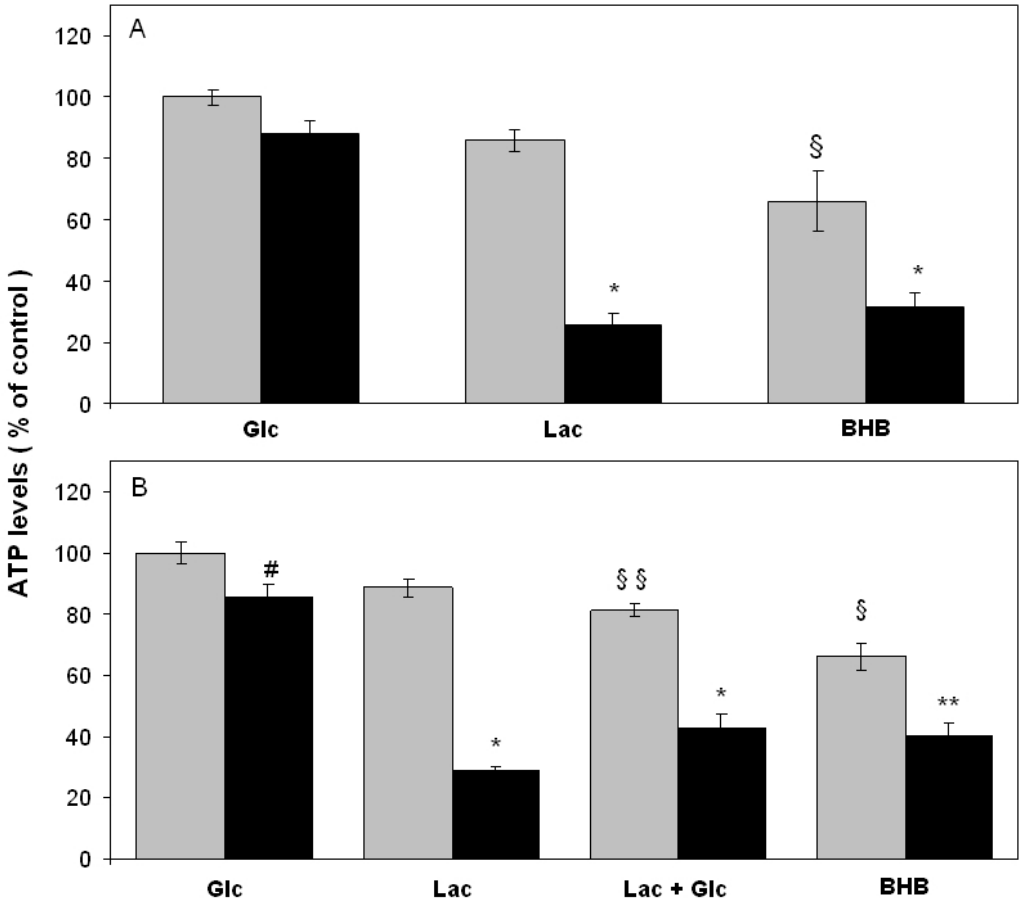

A
B
ATP levels (% of control)
120
100
80
60
40
20
0
Glc
Lac
BHB
Lac + Glc
§
§ §
*
**
#

Fig. 9

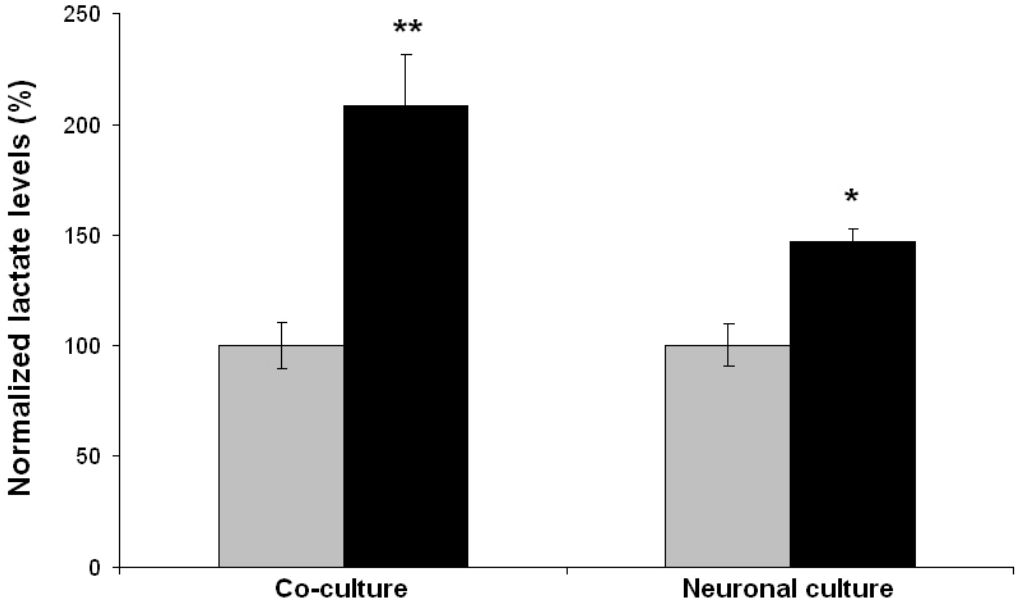
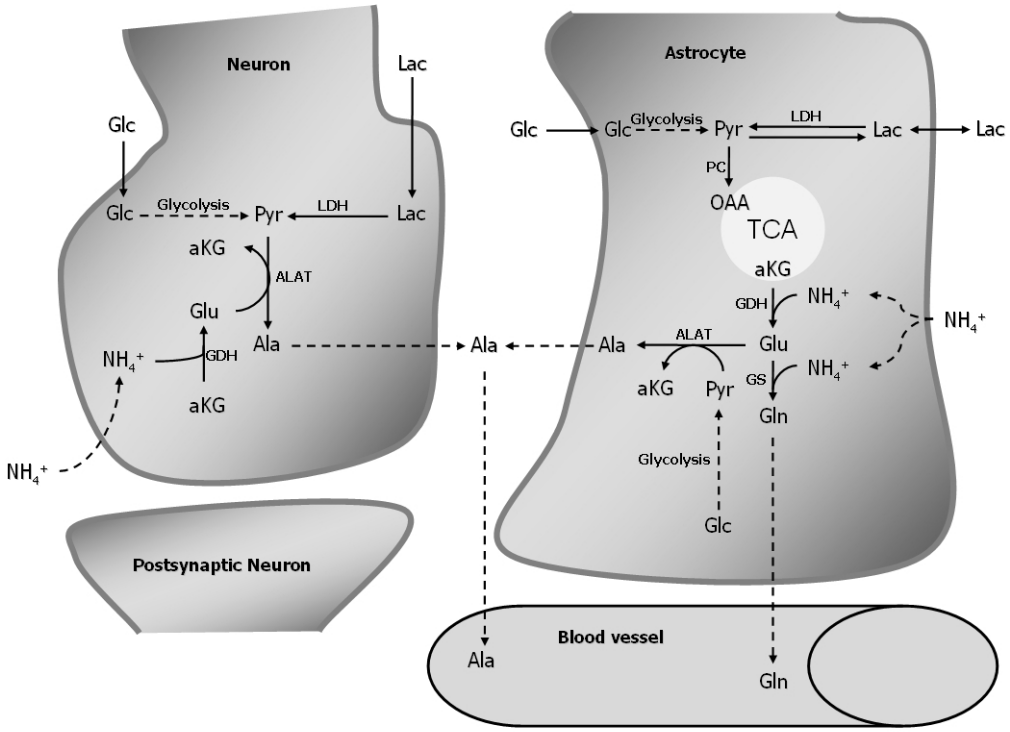

Normalized lactate levels (%)
250
200
150
100
50
0
**
*
Co-culture
Neuronal culture

Fig. 10


Neuron
Lac
Glc
Glc
Glycolysis
Pyr
LDH
Lac
aKG
ALAT
Glu
Ala
NH4+
GDH
aKG
NH4+
Postsynaptic Neuron
Astrocyte
Glc
Glc
Glycolysis
Pyr
LDH
Lac
Lac
PC
OAA
TCA
aKG
GDH
NH4+
NH4+
Ala
Ala
ALAT
Glu
aKG
Pyr
GS
NH4+
Gln
Glycolysis
Glc
Blood vessel
Ala
Gln

CAPÍTULO III

Neuronal GABA synthesis via the tricarboxylic acid cycle is enhanced in bile duct-ligated rats and ammonia-treated co-cultures of GABAergic neurons and astrocytes

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Artigo em preparação

Neuronal GABA synthesis *via* the tricarboxylic acid cycle is enhanced in bile duct-ligated rats and ammonia-treated co-cultures of GABAergic neurons and astrocytes

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ABSTRACT

Hepatic encephalopathy (HE) is a neuropsychiatric disorder which arises from acute and chronic liver disease. It is known that ammonia plays a pivotal role in the etiology of this disease. Moreover, disturbances in the GABAergic neurotransmitter system have been related to HE. Ammonia detoxification from the brain occurs primarily via the enzyme glutamine synthetase and glutamine is important for the replacement of the GABA pool in neurons, being drained by GABA uptake into astrocytes. Hence, the aims of the present study was to evaluate GABA synthesis in co-cultures of neurons and astrocytes in the presence of ammonia (5 mM) and in a rat model of chronic HE induced by bile duct-ligation (BDL). Employing ^{13}C-labeled precursors and subsequent analysis by mass spectrometry of ^{13}C enrichment in selected metabolites, we demonstrate that neuronal GABA synthesis *via* the tricarboxylic acid (TCA) cycle is favored in both BDL animals as well as co-cultures treated with ammonia. GABA synthesized *via* the TCA cycle has been related to the vesicular pool of this neurotransmitter. Further studies in co-cultures revealed that the increased glutamine concentrations were not causally related to the enhanced GABA synthesis *via* the TCA cycle. Therefore, it is likely linked to an increase in the activity of the TCA cycle rather than to elevated levels of extracellular glutamine.

Keywords: Hepatic encephalopathy, GABA synthesis, acetate, glutamine, TCA cycle.

INTRODUCTION

Hepatic encephalopathy (HE) is a neuropsychiatric syndrome with symptoms ranging from mild cognitive impairment to coma, which occurs as a consequence of acute or chronic liver disease (Muñoz, 2008; Hazell & Butterworth, 1999). It is well established that hyperammonemia plays a pivotal role in the etiology, but the exact mechanism is not completely understood (Butterworth, 2002; Felipo and Butterworth, 2002). Increased ammonia concentrations in plasma, cerebral spinal fluid and brain have been related to HE and there is a significant correlation between the plasma ammonia level and the severity of the disease (Clemmesen et al., 1999; Kramer et al., 2000; Butterworth, 2002; Lockwood, 2004).

Almost three decades ago it was suggested that GABAergic neurotransmission activity is enhanced in HE (Schafer and Jones, 1982; Jones and Basile 1998). A generalized central nervous system depression, characterized by impaired motor function and decreased consciousness, is a clear clinical feature in HE (Basile, 2002). In this context, increased GABA release, decreased GABA uptake by astrocytes and increased $GABA_A$ receptor stimulation by the neurotransmitter *per se* or by neurosteroids has been reported (Wysmyk et al., 1992; Oja et al., 1993; Bender and Norenberg 2000; Ahboucha and Butterworth 2007). Interestingly, it appears that ammonia contributes directly and indirectly to some of the events mentioned above (Albrecht and Jones, 1999; Ahboucha and Butterworth 2007).

Ammonia detoxification in the brain occurs primarily *via* the astrocytic enzyme glutamine synthetase (GS) (Norenberg and Martinez-Hernandez, 1979; Schousboe and Waagepetersen, 2007). The enzyme glutamate dehydrogenase (GDH), expressed in both neurons and astrocytes, also participates on brain detoxification from ammonia (Yudkoff et al., 1990; Ott et al., 2005; Schousboe and Waagepetersen, 2007). In

addition, we recently showed that synthesis and release of alanine, produced *via* transamination of glutamate, might be important for disposal of excess ammonia in GABAergic neurons (unpublished data).

In the light of this, increased glutamine synthesis in both cultured astrocytes and co-cultures of neurons and astrocytes exposed to ammonia as well as in animal models of HE has been demonstrated (Chatauret et al., 2002; Zwingmann and Butterworth, 2005). Glutamine has a key function in the so-called GABA-glutamine cycle which operates at the GABAergic synapse to replenish neurotransmitter GABA lost by uptake into the surrounding astrocytes (Bak et al., 2006). In this shuttle, glutamine is synthesized and released by astrocytes and subsequently taken up by neurons where it is deamidated to glutamate by mitochondrial phosphate-activated glutaminase (PAG). In the classical view, glutamate is subsequently decarboxylated to GABA by glutamate decarboxylase (GAD) which enters the neurotransmitter pool. However, it has previously been shown that synthesis of vesicular (i.e. neurotransmitter) GABA involves the TCA cycle to a significant extent (Waagepetersen et al., 1999).

Therefore, we hypothesize that during hyperammonemia, and consequently increased levels of glutamine, the synthesis of GABA might be altered. Hence, we evaluated GABA synthesis in bile duct-ligated (BDL) rats, an animal model of liver cirrhosis leading to chronic HE (Scott-Conner and Grogan, 1994; Butterworth et al., 2009). [U-^{13}C]Acetate were given as metabolic substrates and liquid chromatography-mass spectrometry (LC-MS) technology was subsequently employed to evaluate labeling in GABA as well as glutamate, aspartate and glutamine in the brains of control and BDL rats. In order to obtain information about possible metabolic mechanisms of action at the cellular level, co-cultures of cortical (GABAergic) neurons and astrocytes were exposed to 5 mM NH_4Cl in the presence of [U-^{13}C]acetate or [U-^{13}C]glutamine and

labeling in GABA, glutamate, aspartate, glutamine and alanine were evaluated by LC-MS.

EXPERIMENTAL PROCEDURES

Materials

Seven-day-old NMRI mice and female Wistar rats (mean body weight 200 g; range 192–214 g) aged between 12 and 13 weeks were obtained from were obtained from Taconic (Ry, Denmark). Plastic tissue culture dishes were purchased from NUNC A/S (Roskilde, Denmark), fetal bovine serum from GIBCO, Invitrogen (Taastrup, Denmark). Culture medium and poly-D-lysine (MW > 300,000) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Penicillin was from Leo (Ballerup, Denmark). Isotopically labeled compounds (^{13}C) were all labeled >95% and were either from Cambridge Isotopes Laboratories, Inc. (Massachusetts, U.S.A.) or Isotec (a subsidiary of Sigma Chemical Co.). All other chemicals used were of the purest grade available from regular commercial sources.

Experimental cirrhosis in rats

The experimental cirrhosis in rats was performed as previously described by Bak *et al*, (2009).

Cerebral metabolism of [U-^{13}C]acetate in BDL rats

The rats were fasted for 3 h prior to the experiment. At the time of the experiment they were weighed and then given i.p. injections of 2 mM [U-^{13}C]acetate four times during a 24 min time period. Two min after the last injection, the animals were subjected to inhalation anesthesia and the abdomen was opened. 1 ml blood was taken from the

aorta for measurements of arterial blood ammonia and enrichment of [U-^{13}C]acetate. The rats were decapitated and the brains removed and frozen in liquid nitrogen within 40–50 s after the blood sampling. Within 1 week, the brains were dissected and the forebrain was used for analysis. The tissue samples were extracted in 70% v/v ethanol (ice–cold) and the extracts centrifuged (20,000 g, 20 min). The pellets were dissolved in 1 M KOH and used for determination of protein (Lowry *et al*. 1951). The supernatants were analyzed for labeling in amino acids as described below. Simultaneously with the removal of the brain, muscle tissue samples were frozen in liquid nitrogen. Liver tissue was sampled for histological examination using Haematoxylin & Eosin and Masson's Trichrome staining. Three primary changes for cirrhosis were assessed: disturbed architecture, bile duct proliferation with displacement of hepatocytes, and formation of septa between portal areas containing bile duct cells and connective tissue. According to the degree of these changes in liver architecture, cirrhosis was evaluated as light or severe.

Co-cultures of cortical neurons and astrocytes

Co-cultures of cortical (GABAergic) neurons and astrocytes were prepared as previously described by Leke *et al*. (2008). The cultures were maintained in 6-well culture dishes (35 mm). This co-culture system has been described as a useful tool to study metabolic interactions between astrocytes and neurons, since it exhibits a functional exchange of metabolites (Leke *et al*, 2008).

Incubation experiments

After 7–8 days *in vitro*, co-cultures were pre-incubated in the presence or absence (controls) of 5 mM NH_4Cl for 1h at 37°C. Subsequently, the culture medium was

aspirated and the cultures were rinsed twice in phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 7.3 mM Na_2HPO_4, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, pH 7.4, 37°C). Subsequently they were incubated for 2.5 hours in serum free culture medium (2 ml per well) containing one of the following five different combinations of substrates in the presence or absence of 5 mM NH_4Cl: i) 3 mM [U-^{13}C]acetate, 2.5 mM glucose and 1 mM lactate; ii) 0.1 mM [U-^{13}C]glutamine, 2.5 mM glucose and 1 mM lactate; iii) 0.3 mM [U-^{13}C]glutamine, 2.5 mM glucose and 1 mM lactate or iv) 0.5 mM [U-^{13}C]glutamine, 2.5 mM glucose and 1 mM lactate. The incubation was terminated by removing the medium and washing the cultures twice with ice-cold PBS. Subsequently, the cells were extracted in 70% ethanol and the extracts centrifuged (20,000 *g*, 20 min, 4°C) to separate the soluble extract from the insoluble components. The pellets were employed for protein determination as described above. Cell extracts and incubation media were lyophilized and reconstituted in water for biochemical analysis.

Biochemical analysis

The Phenomenex EZ:faast amino acid analysis kit for LC–MS was used for analysis of labeling in relevant amino acids. Mass spectrometric analysis was performed on an LC–MS system consisting of a Shimadzu LC-MS-2010 mass spectrometer coupled to a Shimadzu 10A VP HPLC system. Protein content was determined according to Lowry *et al*. (1951) using bovine serum albumin as the standard.

Data analysis

Data analysis was performed employing Microsoft Excel 2007 and GraphPad Prism v4.01 software. All labeling data were corrected for natural abundance of ^{13}C by subtracting the mass distribution of a standard containing the relevant metabolites.

Isotopic enrichment was calculated according to Biemann (1962). The average percent of ^{13}C-labeled carbon atoms, i.e. the percent molecular carbon labeling (MCL) was calculated as initially introduced by Bak *et al*. (2006) in glutamate, GABA, aspartate, alanine and glutamine. All data are presented as averages $\pm$ the standard error of the mean (SEM) and differences between groups were analyzed statistically by one-way ANOVA followed by Bonferroni post hoc test. A P value less than or equal to 0.05 was considered statistically significant.

RESULTS

Experimental liver cirrhosis

At six weeks post surgery, bile duct-ligated (BDL) rats exhibited yellowish color of the tail and fur and one third had ascites. Post mortem liver histological analysis confirmed cirrhosis in all BDL rats. Liver weight was significantly higher in the BDL rats (on average 16.8 g, range 10.8-21.3 g) than in the sham-operated rats (on average 8.0 g, range 7.2-10.2 g; $P<0.001$). Both sham and BDL rats gained weight during the post-surgical period. Plasma levels of ammonia were significantly higher in BDL rats (95±16 μmol/l) compared to sham rats (37±7 μmol/l; $P<0.05$).

Labeling patterns in GABA, glutamate, glutamine and aspartate from [U-^{13}C]acetate and [U-^{13}C]glutamine

[U-^{13}C]Acetate is selectively taken up by astrocytes (Waniewski and Martin, 1998) and metabolized to [1,2-^{13}C]acetylCoA. [1,2-^{13}C]acetylCoA is then further metabolized in the astrocytic TCA cycle and will eventually give rise to formation of [4,5-^{13}C]glutamine (i.e. double labeled) derived from [4,5-^{13}C]glutamate labeled from the TCA cycle intermediate α-[4,5-^{13}C]ketoglutarate. [4,5-^{13}C]Glutamine is subsequently

released into the extracellular compartment and taken up by neurons where it is converted to [4,5-^{13}C]glutamate. Subsequently, neuronal [4,5-^{13}C]glutamate can go through one of two different pathways for GABA synthesis: i) it can be decarboxylated directly to [1,2-^{13}C]GABA (referred to as direct synthesis) or ii) enter the TCA cycle *via* transformation to α-[4,5-^{13}C] ketoglutarate and after one full turn of the cycle be converted to α-[3-^{13}C] ketoglutarate (i.e. mono labeled; referred to as *via* the TCA cycle). α-[3-^{13}C] ketoglutarate is then transformed to [3-^{13}C]glutamate giving rise to [3-^{13}C]GABA (referred to as synthesis *via* the TCA cycle; see (Waagepetersen et al., 2001). Thus, double labeled GABA reflects direct synthesis and mono labeled synthesis *via* the TCA cycle. It is important to realize that GABA can only be labeled if labeled glutamine is released from the astrocytic compartment and taken up into neurons where synthesis of GABA takes place. Double labeled aspartate (i.e. [1,2-^{13}C]- or [3,4-^{13}C]aspartate) might be formed from further metabolism of α-[4,5-^{13}C]ketoglutarate in the neuronal or astrocytic TCA cycle and subsequent transamination of the resulting ^{13}C-labeled oxaloacetate to aspartate. [2-^{13}C]- or [3-^{13}C]aspartate will be formed during the second turn of the TCA cycle if labeled oxaloacetate condenses with an unlabeled acetyl-CoA. Triple-labeled isotopomers of glutamate, glutamine and aspartate arise if ^{13}C-labeled oxaloacetate condenses with [1,2-^{13}C]acetyl CoA (astrocytes only, since acetate is only accumulated in these cells (Waniewski and Martin, 1998)) indicating increased TCA cycling rate and/or increased ^{13}C enrichment in the acetyl-CoA pool. When [U-^{13}C]glutamine is employed as substrate, direct neuronal synthesis of GABA *via* [U-^{13}C]glutamate leads to [U-^{13}C]GABA (uniformly labeled) whereas synthesis of GABA *via* the TCA cycle results in [1,2-^{13}C]- or [3-^{13}C]GABA (i.e. mono or double labeled) from [1,2,3-^{13}C]glutamate.

Cerebral metabolism of [U-^{13}C]acetate in BDL rats

BDL animals showed no changes in labeling of glutamate (Fig. 1A), however, significant increases were observed in percent mono labeled GABA and aspartate when compared to the sham group (Fig. 1B,C) indicating a shift in the ratio between direct synthesis of GABA and synthesis *via* the TCA cycle. Labeling in glutamine in the BDL animals was not different from the control animals (Fig. 1D).

Metabolism of ^{13}C-labeled acetate in co-cultures of neurons and astrocytes

In order to obtain more direct information about the effects of ammonia on metabolic aspects of GABAergic neurotransmission, a co-culture system of GABAergic neurons and astrocytes (see Leke et al., 2008) was employed in combination with ^{13}C-labeled substrates acetate (3mM). Incorporation of ^{13}C from acetate (MCL, %) was extensive in glutamine (Fig. 2D) which is in accordance with the notion that acetate is selectively taken up and metabolized in astrocytes (Waniewski and Martin, 1998). In addition, glutamate was considerably labeled (Fig. 2A) approx. 8%). Ammonium chloride did not affect the MCL (%) for any of the four amino acids analyzed when compared to the control cultures (Fig. 2A-D). Labeling from [U-^{13}C]acetate in the different isotopomers of glutamate, GABA, aspartate and glutamine is shown in Fig. 3A-D. Extensive labeling in glutamine (double labeled) and lower but significant labeling in glutamate, GABA and aspartate, all of which are derived from labeled glutamine which to a large extent is transferred from astrocytes to the neuronal compartment in the co-culture system (Leke *et al*., 2008). Interestingly, the cultures exposed to ammonium chloride (5mM) exhibited a significant increase in the percent of double labeled glutamine ($P <$ 0.001) reflecting an increased flux through GS (Fig. 3D). Moreover, the percent of mono and triple labeled glutamine was significantly lower when compared to the

control condition (Fig.3D, $P < 0.001$). Glutamate and aspartate also presented a decrease in the mono labeled isotopomer (Fig.3A and 3C, $P < 0.001$) after ammonium chloride exposure. However, double labeling of glutamate and aspartate did not differ between the control group and the ammonium chloride treated group (Fig. 3A,C). The co-cultures exposed to ammonium chloride also showed a significant decrease in double labeled GABA when compared to the control cultures ($P < 0.01$), showing that GABA synthesis directly from glutamate was diminished (Fig. 3B). On the contrary, ammonium chloride had no effect on the fraction of mono labeled GABA i.e. that formed *via* the TCA cycle (Fig.3B).

Metabolism of ^{13}C labeled glutamine in co-cultures of neurons and astrocytes.

Since the increase in GABA synthesis *via* the TCA cycle is favored in both BDL rats and co-cultures, we hypothesize that this could be due to increased concentrations of glutamine. To test this, metabolism of [U-^{13}C]glutamine at three different concentrations (0.1, 0.3 and 0.5 mM) was investigated. As shown in Fig.4, the MCL of ^{13}C into glutamate, GABA, aspartate and glutamine (Fig. 4A-D) was significantly increased dependent on the [U-^{13}C]glutamine concentration (grey bars, $P < 0.001$). Moreover, the co-cultures incubated with ammonium chloride exhibited a decrease in ^{13}C incorporation in all four amino acids, regardless of the external glutamine concentration (Fig. 4A-D).

In order to evaluate to what extent synthesis of GABA *via* the TCA cycle was affected by ammonium chloride, the labeling in M + 5 (glutamate), M + 4 (GABA) representing direct synthesis without TCA cycle involvement, was compared to labeling in M + 1, M + 2 (GABA) and M + 3 (only glutamate). Fig. 5 shows these results calculated as ratios. In case of glutamate (Fig. 5A), it is seen that the dependency of the TCA cycle

synthesis of the amino acid on extracellular glutamine concentrations, but that the glutamate synthesis *via* TCA cycle is always favored, i.e. ratios smaller than unity. Exposure to ammonium chloride significantly decreased the ratio at 0.1 mM [U-^{13}C]glutamine (Fig.5A, $P < 0.05$). Concerning the analogous ratios for GABA synthesis, values were equal and slightly higher than unity at 0.1, 0.3 and 0.5 mM of [U-^{13}C]glutamine, showing that the pathway by which GABA was synthesized is independent of the extracellular concentration of glutamine and exposure to ammonium chloride and that TCA cycle-dependent GABA synthesis equals that representing direct synthesis from glutamine (Fig. 5B).

DISCUSSION

The increased GABAergic tone hypothesis during HE has been suggested in the early eighties based on experiments performed in rabbits with fulminant hepatic failure which showed similarity in evoked potentials with those caused by $GABA_A$ agonists (Jones and Basile, 1998). Subsequent to these observations, several studies were undertaken to elucidate possible mechanisms of the increased GABAergic tone in HE. Several studies were performed in order to evaluate GABA concentrations during HE and it is commonly agreed that GABA levels are unchanged in both brain and cerebrospinal fluid of patients with HE, as well as in brain tissue from animal models of chronic and acute HE (Ahboucha and Butterworth, 2004). However, decreases in GABA transporter activity and reductions in $GABA_B$ receptor expression, which regulates vesicular GABA release, were demonstrated in different systems (Wysmyk et al., 1992; Oja et al., 1993; Bender and Norenberg 2000). Both phenomena together would lead to an increase in GABA available for $GABA_A$ receptor interaction. In addition, $GABA_A$ receptor integrity was found to be unaltered during this pathology

(Ahboucha et al., 2003; Ahboucha and Butterworth, 2005). However, recent studies showed changes in $GABA_A$ receptor subunit expression in models of HE and hyperammonemia (Li et al., 2005; Cauli et al., 2009). Hence, it has been demonstrated that ammonia directly stimulates the $GABA_A$ receptor (Takahashi et al., 1993; Ha and Basile, 1996) and that the increased production of certain neurosteroids also potentiates GABA neurotransmission (Ahboucha and Butterworth, 2007)

In the present study, GABA synthesis was evaluated in a rat model of chronic HE (i.e. BDL rats), as well as in co-cultures of cortical neurons and astrocytes. In both models it was verified that GABA was preferentially synthesized *via* the TCA cycle as compared to the direct synthetic route not involving the TCA cycle. The cirrhotic rats with chronic HE, which had increased blood levels of ammonia, exhibited an increase in GABA synthesized *via* the TCA cycle when [U-^{13}C]acetate was the labeled substrate. The GABAergic co-culture system exhibited a decrease in the direct synthesis of GABA and no alteration in its synthesis involving the TCA cycle, after incubation with the same labeled substrate together with ammonium chloride. Although not exactly presenting the same pattern, the overall result is that GABA synthesis occurred preferentially *via* the TCA cycle in both experimental models. Contrary to the results presented here, a study employing the model of acute liver failure in rats by portacaval anastomosis and NMR technology did not observe any change in *de novo* synthesis of GABA either at precoma or coma stages of HE (Zwingmann et al., 2003).

It has been shown that GABA synthesis to a large extent involves glutamine metabolism through the TCA cycle and that this pool of GABA constitutes the vesicular neurotransmitter pool (Waagepetersen et al., 1999; Waagepetersen et al., 2001). In agreement with the presence of distinct pools of GABA (i.e vesicular and cytoplasmic), the enzyme GAD is expressed in brain as two different isoforms, GAD_{67}

and GAD_{65}. GAD_{67} is found widespread throughout neuronal cell bodies whereas GAD_{65} is primarily localized in axon terminals (Stone et al., 1999). Furthermore, it is likely that GAD_{65} is related to synthesis of vesicular GABA, while GAD_{67} is fundamental for cytoplasmic GABA synthesis and also plays an important role on vesicular GABA synthesis (Tian et al., 1999; Waagepetersen et al., 2001). Given this, the favored GABA synthesis *via* the TCA cycle described in the present study might lead to an increase in the vesicular GABA pool size.

One hypothesis that could explain this favored GABA synthesis *via* the TCA cycle during ammonia-induced toxicity is the increase in glutamine synthesis and release by astrocytes. Thus, raised glutamine levels would be available for neuronal uptake, which could influence GABA synthesis pathways (i.e. with or without TCA cycle involvement). To verify this possible mechanism, the co-cultures were incubated with three different concentrations of ^{13}C-labeled glutamine. The results showed that increased incorporation of ^{13}C in the amino acids glutamate, GABA, aspartate and glutamine was dependent on the exogenous [U-^{13}C]glutamine concentration as previously shown by Leke et al. (2008). Moreover, exposure to ammonia decreased the ^{13}C incorporation in all amino acids, including GABA, which is likely to be a consequence of enrichment by ^{13}C of newly synthesized, unlabeled glutamine due to PC activity. The effect of ammonia has been shown to increase PC activity and glutamine synthesis in different models of HE (Kanamatsu and Tsukada, 1999; Zwingmann et al., 2003; Zwingmann, 2007). In addition, glutamate synthesis from glutamine occurred preferentially *via* the TCA cycle (Leke et al., 2008). On the contrary, GABA synthesis appeared to be independent of the increasing glutamine concentrations as well as ammonia exposure, being mediated equally *via* the TCA cycle and *via* direct synthesis. These results likely represent a compartmentalized glutamate

pool, a phenomenon that has been described previously (Westergaard et al., 1995). It has been shown that glutamine metabolism in cultured neurons lead to enhanced glutamate formation *via* the TCA cycle, whereas it had no effect on GABA metabolism (Waagepetersen et al., 2000). Recently, the same pattern of compartmentalization of GABA metabolism was demonstrated in mouse cerebral cortical mini-slices superfused in media containing labeled glucose and glutamine (Waagepetersen et al., 2008).

The results described here so far indicate that GABA synthesis *via* the TCA cycle is enhanced during HE and hyperammonemia which is independent upon rasing exogenous glutamine concentrations. One possible mechanism which could explain the increased GABA synthesis would be an enhancement of TCA cycle activity. In fact, we have recently shown that hyperammonemia increases glycolysis and TCA cycle metabolism in cultures of cortical neurons (unpublished data), a result compatible with that of a former study performed in cultured cerebellar neurons (Johansen et al., 2007). Therefore, an increased TCA cycle activity would lead to and increase in the GABA synthesis related to the vesicular pool. However, to what extent the TCA cycle enhancement is the only mechanism responsible for the increased GABA synthesis *via* the TCA cycle is still unclear. It should also be considered that ammonia may have an effect on the enzymes regulating the GABA metabolism. Previous findings showed unchanged activity for GAD in patients and animal models of liver failure (Ahboucha and Butterworth, 2004). However, total GAD activity might not represent the GABA synthesis in close connection with the vesicular pool. Further experiments are necessary to better clarify the effects of HE and ammonia on the specific isoforms of GAD and other enzymes which regulate GABA metabolism.

In conclusion we demonstrate that GABA synthesis is affected in both *in vivo* and *in vitro* models of HE and, although very important for GABA synthesis, glutamine

availability apparently is not the reason why GABA is preferentially synthesized *via* the TCA cycle. We hypothesize that the increased GABA synthesis could be a consequence of increased TCA cycle activity. To what extent the increase in GABA synthesis, which is known to be connected to the vesicular GABA pool, is important for the enhancement in the GABAergic tone awaits further studies. Moreover, the changes observed in GABA synthesis described here might also be important for understanding the behavioral impairments associated with HE. Therefore, the BDL rat model of HE may be a relevant model for the study of different behavioral parameters.

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LEGENDS

Figure 1: Labeling (%) in glutamate (A), GABA (B), aspartate (C) and glutamine (D) in forebrain extracts from sham operated (n=6, grey bars) and BDL rats (n=6, black bars) treated with four injections of 3 mM [U-^{13}C]acetate. Results are presented as averages ± S.E.M. (*) $P < 0.01$ by ANOVA followed by Bonferroni post hoc test when comparing the control group with the BDL group.

Figure 2: Average incorporation of ^{13}C (MCL, %) in glutamate (A), GABA (B), aspartate (C) and glutamine (D) in co-cultures of cortical neurons and astrocytes incubated in serum-free culture medium containing 3 mM [U-^{13}C]acetate, 2.5 mM glucose and 1 mM lactate in the presence or absence of 5 mM ammonium chloride for 2.5 h. The ammonia group was submitted to a pre incubation of 1h in a serum-free culture medium with 5 mM ammonium chloride. Results are averages ± S.E.M. of 4–5 cultures. The cultures exposed to ammonia are represented as black bars and the control condition as grey bars.

Figure 3: Labeling (%) in glutamate (A), GABA (B), aspartate (C) and glutamine (D) in co-cultures of cortical neurons and astrocytes incubated in serum-free culture medium containing 3 mM [U-^{13}C]acetate, 2.5 mM glucose and 1 mM lactate in the presence or absence of 5 mM ammonium chloride for 2.5 h. The ammonia group was submitted to a pre incubation of 1h in a serum-free culture medium with 5 mM ammonium chloride. Results are averages ± S.E.M. of 4–5 cultures. Statistical analysis was performed by ANOVA followed by Bonferroni post hoc test. (*) $P < 0.01$ when comparing the cultures exposed to ammonia (black bars) with the control condition

(grey bars). (**) $P < 0.001$ when comparing the cultures exposed to ammonia (black bars) with the control condition (grey bars).

Figure 4: Average incorporation of ^{13}C (MCL, %) in glutamate (A), GABA (B), aspartate (C) and glutamine (D) in co-cultures of cortical neurons and astrocytes incubated with three different concentrations of glutamine (0.1, 0.3 and 0.5 mM) in the presence or absence of 5 mM ammonium chloride for 2.5 h. The ammonia group was submitted to a pre incubation of 1h in a serum-free culture medium with 5 mM ammonium chloride. Results are averages ± S.E.M. of 4–5 cultures. Statistical analysis was performed by ANOVA followed by Bonferroni hoc test. (*) $P < 0.05$ when comparing the cultures exposed to ammonia (black bars) with the respective control condition (grey bars). (**) $P < 0.05$ when comparing the 0.3 mM and 0.5 mM to 0.1 mM glutamine control groups. (#) $P < 0.05$ when comparing the 0.3 mM control condition to 0.5 mM control condition group.

Figure 5: Ratios of M + 5 (glutamate, A) or M + 4 (GABA, B) divided by the sum of (M+ 3, M+ 2, M+ 1) and (M+ 2, M+ 1), respectively, in co-cultures cortical neurons and astrocytes incubated with three different concentrations of glutamine (0.1, 0.3 and 0.5 mM) in the presence or absence of 5 mM ammonium chloride for 2.5 h. The ammonia group was submitted to a pre incubation of 1h in a serum-free culture medium with 5 mM ammonium chloride. Results are averages ± S.E.M. of 4–5 cultures. Statistical analysis was performed by ANOVA followed by Bonferroni hoc test. (*) $P < 0.05$ when comparing the cultures incubated with 0.1 mM glutamine with the 0.3 and 0.5 mM groups. (**) $P < 0.05$ when comparing the cultures exposed to ammonia (black bars) with the control condition (grey bars).

Fig. 1

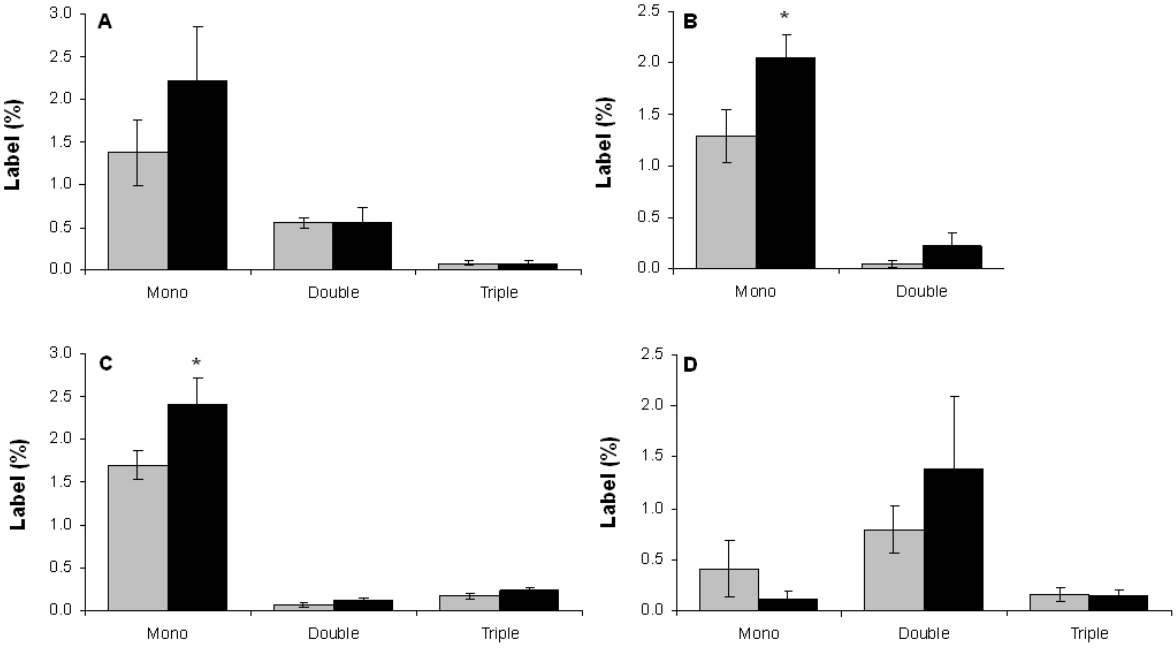

Fig. 2

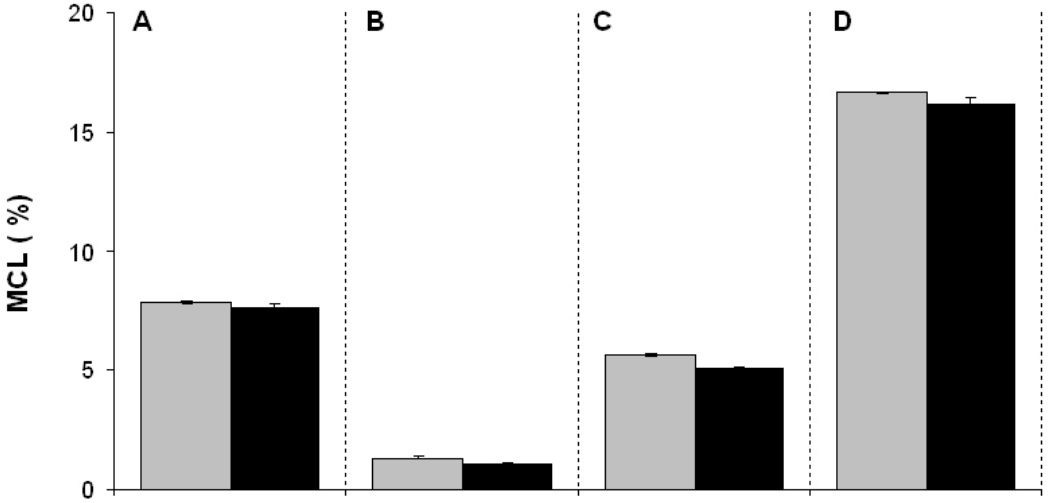

Fig. 3

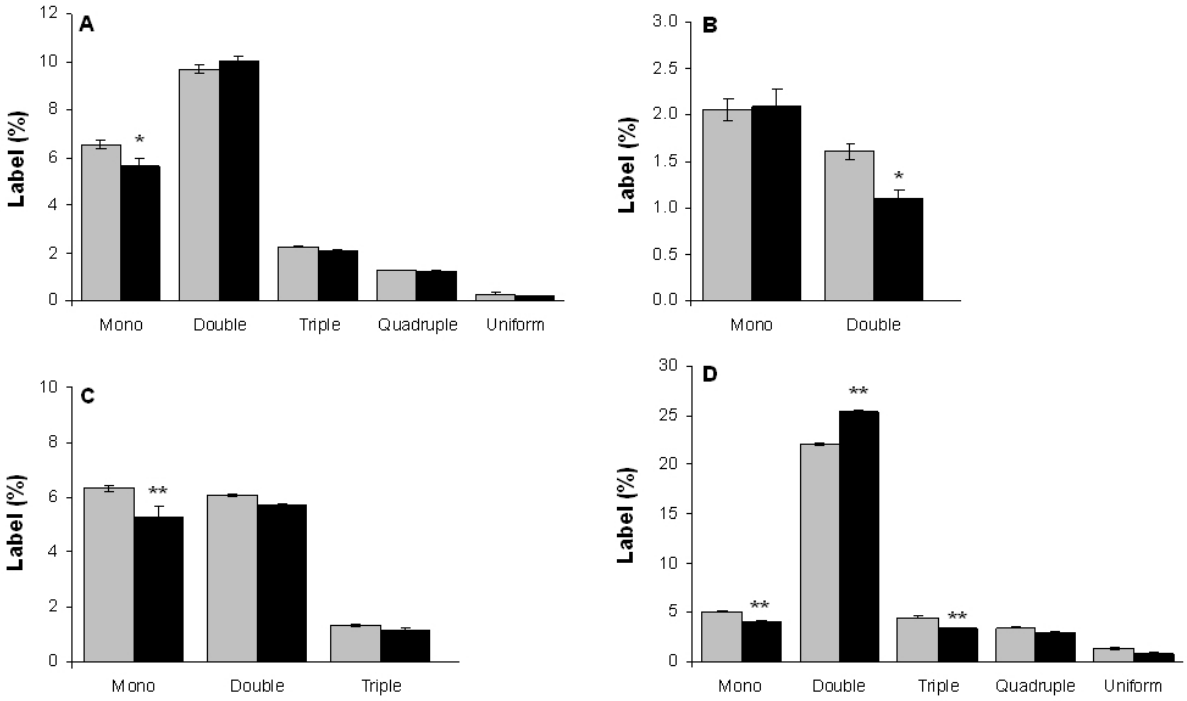
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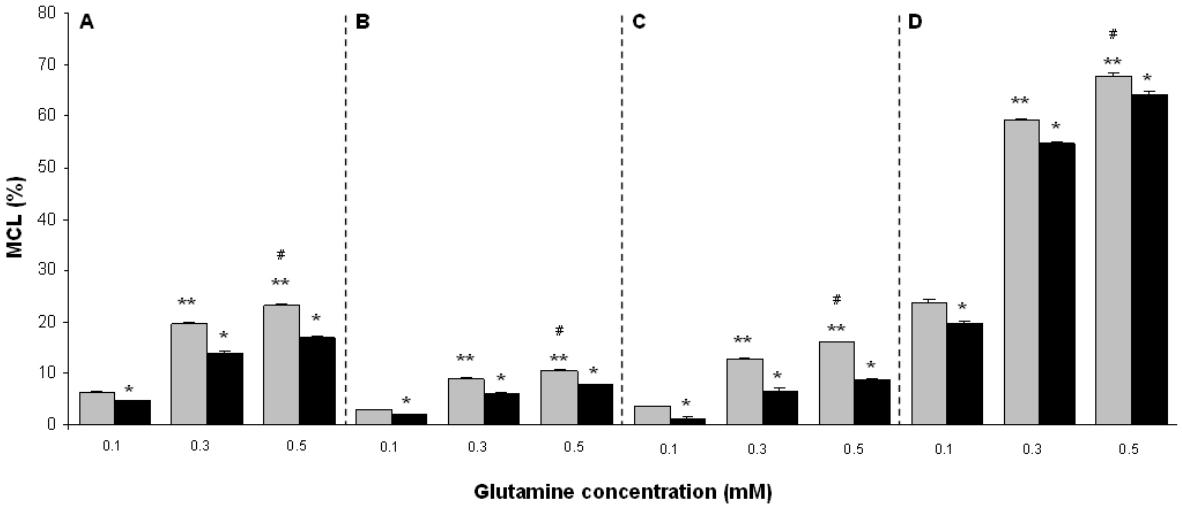
Fig. 4


A
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MCL (%)
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40
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80
0.1
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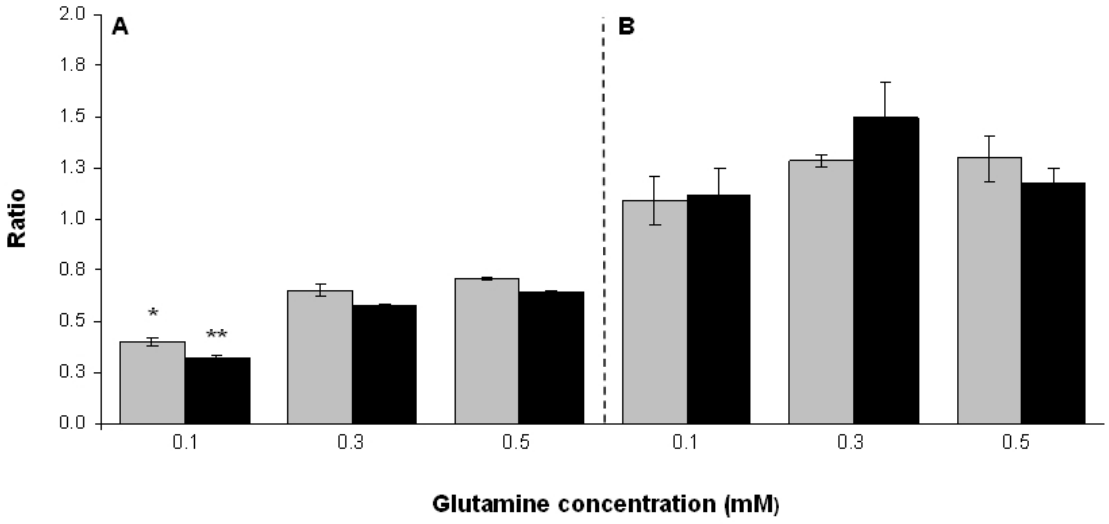
Glutamine concentration (mM)

Fig. 5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Ratio
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0.1
0.3
0.5
0.1
0.3
0.5
Glutamine concentration (mM)

CAPÍTULO IV

Spatio-temporal locomotor and exploratory activities impairment and object recognition memory deficits in rats with hepatic encephalopathy induced by bile duct ligation.

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Artigo em preparação a ser submetido ao periódico Journal of Hepatology

Spatio-temporal locomotor and exploratory activity impairment and object recognition memory deficits in rats with hepatic encephalopathy induced by bile duct ligation.

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Abstract

Hepatic encephalopathy (HE), a disorder which arises from liver failure, leads to several problems including motor and cognition impairment. Bradykinesia and hypokinesia are common symptoms observed in patients with HE and moreover they also exhibit memory deficits. Animal models of HE have been extensively employed in order to study the mechanisms behind this disease. Impairment in locomotor activity and memory has been described in different rat models of HE; however, studies may be controversial. Therefore, the aim of this study was to evaluate the locomotor and exploratory activity and memory in a rat model of HE type C, produced by bile duct ligation (BDL). Here we show that BDL rats exhibit altered spatio-temporal locomotor and exploratory activities which were not a result of anxiety behavior or motor coordination impairment. Moreover, BDL rats exhibited short term memory deficits when performing the object recognition task. Therefore, the BDL rat model appears to be a good model to study type C HE since it truly represents the pathological mechanisms and also the clinical manifestations of HE. The neurochemical mechanisms and brain areas which are involved in hypolocomotion and cognition impairment are still not completely understood and further studies are necessary to elucidate this.

Keywords: Hepatic encephalopathy, bile duct ligated rats, hypolocomotion, memory, open field, plus maze, object recognition.

Introduction

Hepatic Encephalopathy (HE) is a neuropsychiatric disorder which arises from acute or chronic liver diseases [1,2]. According to the etiology of liver injury, HE is classified as follows: type A, derived from acute liver failure; type B, related to hepatic portal-systemic shunts without hepatocellular disease; type C, associated with cirrhosis and portal hypertension or portal-systemic shunts [3]. Moreover, type C HE is subclassified according to duration and characteristics of neurologic manifestations as episodic, persistent and minimal HE [3].

The clinical manifestations of HE depend on the degree of metabolic disturbances and the rate in which organ dysfunctions occur. Therefore, the symptoms can range from sleep disturbances and slight attention deficits to somnolence-sopor and coma [4]. HE patients experience motor function impairment, such as bradykinesia, hypokinesia and ataxia. Different studies have also described altered locomotor activity in animal models of HE. Although the majority has shown decreased spontaneous locomotor activity, some results are controversial [5]. Moreover, HE patients also exhibit attention deficits and memory and learning impairment [4,6]. These cognitive alterations were also reproduced in animal models of HE and decreases in performance were observed for tasks evaluating learning ability as well as spatial and working memories [7,8,9,10].

The bile duct ligated (BDL) rat is a model of type C HE where cirrhosis is induced due to obstruction of the common bile duct [11,12]. The BDL rat model is very reproducible and truly represents the pathophysiology mechanisms of liver disease present in HE patients [11]. Although considered a good model to study HE, it has not been largely used in comparison to other animal models, such as the portocaval anastomosis. But in contrast to the latter BDL rats develop liver failure, jaundice, portal hypertension and portal-systemic shunting [11].

On this basis, the aim of the present study was to investigate the locomotor and exploratory behavior in BDL rats as well as to access the object recognition memory in order to verify if this kind of memory was altered by HE.

Methods

Subjects

Adult female Wistar rats (weight 180 ± 13 g, 60 days of age at the start of the surgical procedure) were obtained from the animalarium of Biochemistry Department (Federal University of Rio Grande do Sul). They were housed 3 per cage and kept in a controlled environment (room temperature of 21±1°C, standard light/dark cycle of 12 h) with standard food and water *ad libitum*. The handling and care of animals were conducted according to the guidelines of the Guide for the Care and Use of Laboratory Animals, NIH (USA). All procedures were approved by the Ethics Committee from the Federal University of Rio Grande do Sul.

Bile duct ligation

The bile duct ligation procedure was conducted according to Bak et al [13] with minor modifications. Rats were anaesthetized with ketamine (90 mg/kg) and xylazine (12 mg/kg) i.p. and placed in the supine position on an operating table and to assure constant body temperature during the surgical procedure a thermal controlled mattress (37°C) was used. A middle abdominal incision was made and the hepatic ligament was exposed. The common bile duct was ligated by two ligatures with 4-0 non absorbent surgical suture, the first being made below the junction of the biliary hepatic ducts and the second above the entrance of the pancreatic ducts. Subsequently, the common bile duct was resected between the two ligatures. The abdominal incision was closed with 4-

0 non absorbent suture in two layers and the rat was placed in another thermal mattress until regain of consciousness and subsequently returned to its home-cage. The control group consisted of Sham-operated rats which had the hepatic ligament exposed and manipulated, but not ligated. All animals received a subcutaneous injection of ketoprofen (1 mg, Merial, Brazil) and benzylpencyline (35 mg, Fort Dodge Saúde Animal LTDA, Brazil) during 5 and 7 days after surgical procedures, respectively, and were kept in the animal colony room during the following 6 weeks.

Behavioral studies

All experiments were conducted between the sixth and seventh week after surgery procedure. During the time between postsurgery and the beginning of behavior tasks the rats were weekly manipulated to monitor weight gain and to avoid stress. Before each behavior task the rats were placed in the test room for one hour to allow habituation with the environment and researchers. Inter-task intervals were 2 days and all behavioral tasks were performed between 9:00-16:00 h. All behavioral parameters were recorded and analyzed by the video tracking system Any-maze^{TM} (Stoelting, USA).

Open field

In order to evaluate the locomotor and exploratory activities, animals were placed into a 60 cm diameter circular black arena (walls 50 cm high) facing the wall and left free to explore it for 15 min. The area was divided in two main sections (center and periphery) and the room was illuminated by two halogen lamps (100 lx) pointed towards the room walls. After each trial, the apparatus was cleaned with an ethanol solution (30%). The following parameters were quantified: (1) locomotor activity (total distance traveled,

time mobile/immobile, number of stops, inter stop distances, total number of rearing and grooming); (2) temporal organization of activity (number of trips, trips length and the ratio between stops per trip); and (3) spatial distribution of locomotion (distance traveled along the center of the arena, calculated as percentage of the total distance traveled; and time spent locomoting in the center of the arena, calculated as percentage of the total locomoting time).

Elevated Plus Maze

Anxiety behavior was assessed by the elevated plus maze which relies on the rodent's proclivity towards dark, enclosed spaces and an unconditioned fear for heights and open spaces [14]. The apparatus consisted of four 50 cm long and 10 cm wide arms, two of which contained 40 cm high walls opposite each other. The apparatus was elevated 50 cm on the floor and situated in the center of a red lit room (60 lx). Rats were placed individually at the center of apparatus and left free to explore it for 5 min. After each trial, the apparatus was cleaned with an ethanol solution (30%). The following parameters were quantified: (1) number of entries in the open and closed arms; and (2) time spent on the open and closed arms.

Foot-fault

In order to verify coordinated fore and hind limb placement during spontaneous exploration rats were placed on a grid and the number of failed attempts to accurately grasp the rungs was analyzed. The apparatus consisted of an elevated wire grid platform 80cm x 60cm, 76.5-cm above the floor with 3 cm^2 holes. The animals were allowed to freely explore for 3 min. A foot-fault was scored when the rat misplaced its paw and the limb fell between the rungs. Total number of foot-faults was quantified.

Object Recognition

The object recognition task was performed according to the protocol described by Bevins and Besheer [15]. The object-recognition apparatus consisted of a black wooden square arena (50cm×50cm×50cm) placed in a room where the light intensity (100 lx) was equal in the different parts of the apparatus. Four objects (two identical and two different) made of inert material that could not be displaced by the rat were used for discrimination. In order to habituate animals, a session of 5 minutes in the empty arena was performed 24h before the task. The object recognition task consisted of a training and two test sessions, all of which of 5 min duration. In the training session rats were individually placed into the arena and exposed to two identical objects (A and B). Rats were then removed and returned to their home-cage. In short-term memory (STM) evaluation, animals were placed into the arena 3h later and exposed to the familiar object A and a novel object C. To assess long-term memory (LTM), rats were exposed to the familiar object A and another novel object D 24h after training session. Recognition was defined as sniffing or touching the object with the nose and/or forepaws. In all sessions the animals were placed in the open arena facing away from the objects and the objects were cleaned with 30% alcohol between trials. The time spent to explore each object was expressed as a percentage of the total time exploring both objects.

Samples collection

Rats were anesthetized with ketamine (90 mg/kg) and xylazine (12 mg/kg) i.p. and blood was withdrawal by cardiac puncture. Animals were decapitated and the brain was dissected and stored in a solution of 10% formaldehyde solution (10% formaldehyde in a solution of 0.1 mM phosphate buffer containing 21.6 mM Na_2HPO_4 and 81 mM

NaH_2PO_4, pH 7.4). In order to collect organ biopsies for histopathology, a middle abdominal incision was made, ascitic fluid was collected when present, and liver and spleen were carefully removed, weighed and stored in a 10% formaldehyde solution. All organs were stored at 4°C until histological analysis. Blood samples were centrifuged for 5 minutes at 5000×g and 20 °C and the serum and ascitic fluid were stored at -20 °C until analysis.

Histopathological examination

First, liver and spleen from control and BDL groups were examined macroscopically. Subsequently, liver was embedded in paraffin and micron sections were taken randomly from the left and right lobe. These micron slices were mounted and stained with hematoxylin-eosin (H&E) and picrosirius.

Biochemical parameters

Plasma samples from the control and BDL groups were analyzed for biochemical parameters, i.e. aspartate aminotransferase (AAT), alkaline phosphatase, bilirubin (total and direct), and albumin, using an auto-analyzer Cobas Integra 400 (Roche Diagnostics Corporation®).

Statistical analysis

Data were expressed as mean ± SEM. Data from biochemical determination, open field and foot fault were analyzed by Studet's t-test. To object recognition and elevated plus maze, data were analyzed by two-way ANOVA followed by Bonferroni post hoc test. For all parameters, $p < 0.05$ was considered significant.

Results

Experimental cirrhosis

Bile duct ligated rats (BDL) exhibited yellowish color of the fur and tail, an enlarged abdomen and all of them had ascites. The body weight was not different between the control (224 ± 18 g) and BDL (226 ± 21 g) groups at the sixth week after surgery procedure.

Biochemical and histopathological characterization

Hepatomegaly and splenomegaly were observed in the BDL rats, liver and spleen weight was significantly higher in the BDL rats (26.67 ± 2.47g, 2.61 ± 0.24g, respectively) than in the control rats (7.83 ± 0.20g, 1.14 ± 0.10g, respectively $p<0.05$). Moreover, BDL rat's livers were tinged with yellow and presented a cystic duct remnant, developed from the bile duct remnant, containing viscous fluid. Histological analysis of the liver sections stained with H&E showed marked ductal proliferation, especially around the portal area, for the BDL rats when compared to the control group (Fig.1A-B). The analyses for the picrosirius staining, which reveals the collagen fibers, demonstrated an noticeable increase of collagen fibers which extended from portal to portal areas as well as from the portal area to the centrilobular venule in the BDL samples, whereas the control group has its hepatic parenchyma with the normal histology (Fig 1C-D).

The plasma analysis showed that the activities of ATT and alkaline phosphatase were significantly higher for the BDL rats as well as the amounts of bilirubin (total and direct) (Table 1, $p<0.01$). Albumin concentrations were decreased in the BDL rats when compared to the control group and it was verified the presence of albumin in the ascitic fluid from the BDL rats (0.67 ± 0.36 g/dL).

Behavioral studies

Open field

Locomotor activity

BDL rats exhibited a significantly smaller total distance traveled in the open arena and a higher duration on immobile episodes, and consequently a smaller duration on mobile episodes, when compared to the control group (Fig 2A-B). When analyzing the distance traveled across time it was verified that both groups presented the same pattern of behavior, traveling larger distances at first minutes of the task and subsequently decreasing the locomotor activity, although the BDL group exhibited significantly smaller distances traveled in the first 6 minutes when compared with the control group (Fig 1A suppl.). The same pattern of behavior was observed in mobility across exploration time: BDL rats presented significantly less time being mobile during the first 7 minutes of exploration when compared to the control group, however, after this time period both groups exhibited the same pattern of behavior (Fig 1B suppl.). The total number of stops during the exploratory activity was not different between the BDL and control groups (Fig 2C), albeit, the distance traveled between these stops was significantly smaller for the BDL group (Fig 2D). The total number of rearing performed during the task was significantly decreased in the BDL group and analysis across time showed that this difference was only apparent during the first two minutes of exploration (Fig. 1C suppl.). Grooming behavior did not differ between the groups (Fig. 2E).

Temporal organization of behavior

There was a significant increase in the time period that the BDL animals stayed in the home-base area when compared to the control group (Fig 3A). The number of trips

performed during the task was not different between both groups (Fig 3B), however, the length of each trip was considerably shorter for the BDL group (Fig 3C). The number of stops made in trips between two successive stops at the home-base (stops/trips) was not different between the groups (Fig 3D).

Spatial distribution of activity

The percentage of distance traveled along the center zone was not different between the BDL and control group (Fig. 4A). However, the percentage of time spent in the center of the arena was significantly smaller for the BDL group when compared to the control group (Fig 4B).

Plus maze

There was no difference in the total number of entries made on open and closed arms between the BDL and control groups, however, the BDL rats exhibited fewer entries in both arms when compared to the control group (Fig 5A). Concerning the time spent in the arms, both groups stayed approximately the same period of time in the open and closed arm (Fig 5B).

Foot-fault

The was no difference in the total number of foot-faults performed by the BDL rats when compared to the control group (Fig. 6)

Object Recognition

The BDL and control groups showed the same pattern of behavior during the training session, as both spent the same period of time recognizing the objects A and B (Fig 7).

When evaluating STM, the BDL group did not show difference in the discrimination index for objects A and C, whereas the control group exhibited a significantly increased discrimination index for object C when compared to object A. In the last session for LTM, both groups had a significantly increased discrimination index for object D when compared to object A.

Discussion

In the present study it was demonstrated that BDL rats exhibited impaired locomotor and exploratory activities in the open field task, characterized by a decreased total distance traveled and increased period of time being immobile. Moreover, the BDL rats traveled shorter distances between stops during the exploratory activity, although the total number of stops was not different between the control and BDL groups. Reinforcing the concept of altered locomotor and exploratory activities, the total number of rearing was smaller for the BDL rats. These data are in accordance with previous studies employing different animal models of HE all demonstrating locomotor dysfunction. Different authors have shown that rats subjected to portacaval anastomosis (PCS), a model of type B HE, exhibit hypolocomotion in the open field task [16,17,18]. Another study which was conducted employing the BDL model also demonstrated that the cirrhotic rats exhibited a decreased locomotor activity when subjected to the open field task [19]. Conversely, Méndez et al. [9] did not observe differences in locomotor behavior when studying the PCS or the thioacetamide-induced HE.

It is important to note that the majority of studies which verified the locomotor and exploratory activities during HE analyzed basically the total distance traveled or number of crossings in the arena, and total number of rearing. In the present study the open field task was more explored in more detail since temporal and spatial activity as

well as the organization of exploratory and locomotor activities were also investigated. It is well known that rats after being introduced to the open field establish a home base, which is the most visited location of the arena where they spend an extended period of time and is the place from which the rats will initiate trips [20]. Further analysis of the temporal (sequential) structure of locomotor behavior of the BDL rats showed that these animals remained in the home base area for longer periods of time than the control group and both groups performed the same number of exploratory trips. Therefore, the decreased total distance traveled by BDL rats in the open field does not appear to be related either with the number of trips or with the number of stops per trip performed by the animals. However, the distance traveled per trip was smaller for the BDL group, which consequently led to a reduction in the total distance traveled (See Fig. 8). With regard to the spatial distribution of locomotion, the BDL rats did not differ from the control group in the percentage of distance traveled in the center zone of the arena. However, the time spent in this area was decreased for the BDL rats. This pattern of behavior could be justified as avoidance of the open area which has been related to an anxiety-like behavior. For this reason, the elevated plus maze task was conducted and it was verified that the BDL rats did not exhibit anxiety behavior. However, the BDL rats showed a decreased total number of entries made in both open and closed arms when compared to the control group, while the time spent in the arms was not different between the groups. These data indicate that the cirrhotic rats entered less times and remained longer periods in the closed and open arms. Therefore, the BDL rats exhibited the same pattern of behavior of altered locomotor-exploratory activity presented previously in the open field apparatus, which was not a consequence of an anxiety behavior. Furthermore, the diminished locomotor activity exhibited by the BDL rats was not a consequence of impaired motor coordination because the BDL rats

were as coordinated as the control group when performing the foot fault task. According to this, Sergeeva et al. [21] did not observe anxiety behavior in rats subjected to PCS, while the animals moved less and more slowly in the open field task when compared to the control group. In addition, rats with chronic liver failure induced by thioacetamide intoxication did not show anxiety behavior, but it should be noted that they did not demonstrate locomotor and exploratory deficits either [9]. Taken together, our results clearly demonstrate that the BDL rats had altered spatio-temporal organization of locomotor and exploratory activities, but the hypolocomotion observed did not appear to be the result of anxiety or impairment in motor coordination. Impairment of the motor system has been extensively studied in HE patients. It has been described that hypokinesia and bradykinesia observed in HE patients are a consequence of altered basal ganglia function [22,23] (Jover et al., 2005; Giewekemeyer et al., 2007). Moreover, it also has been suggested that the motor alterations observed in HE are related to altered functional connections between the basal ganglia-thalamo-cortical loop [17]. The mechanisms by which these cerebral areas are affected during HE are still unclear albeit several studies have been undertaken to elucidate these mechanisms. Joebges et al. [24] have shown that the cortical brain areas such as cyngulate gyrus, frontomesimal and parietal cortex, which are related to movement initiation, exhibited decreased glucose uptake in HE patients. Moreover, a study employing the PCS rat model demonstrated an increase in extracellular glutamate and metabotropic glutamate receptor mGluR1 activation in substantia nigra reticulata which may be related to the reduced motor activity observed in these animals [16]. The activation of an alternative neuronal circuit was also shown in PCS rats, as a result of altered dopaminergic and glutamatergic neurotransmission, which could explain the locomotor problems observed [17]. In addition, increased

levels of neurosteroids and consequently dysfunction in the GABAergic neurotransmitter system might also participate in the altered locomotor activity observed in PCS rats [18].

Besides having motor activity impairment, HE patients also exhibit cognitive dysfunctions such as memory deficits [4,25]. In order to evaluate cognitiove function in BDL rats the object recognition task was performed, a task which relies on the natural tendency of rodents to explore preferentially novel stimuli over familiar stimuli [26]. In the training session both BDL and control groups behaved similarly, exploring the two identical objects for the same period of time. This result is very important since it reveals that the BDL rats were as motivated as the control group when facing the novelty. In addition, the BDL rats showed impaired STM, whereas the LTM was unaffected. Contrary to the results presented here, a study by García-Moreno et al. [27] described impaired LTM in BDL rats. However, it should be considered that the protocol employed in this study was different from the one described here, i.e. the BDL rats were not tested for STM and the delay for the LTM session was 48 hours. With regard to the brain areas involved in the object recognition memory, it has been described that the medial temporal lobe, constituted by hippocampus, entorhinal, perirhinal and parahippocampal cortices, is responsible for the mediation of declarative memory processes [26]. However, recent findings have questioned the importance of the hippocampus in object recognition memory and it appears that the perirhinal cortex plays a more important role in this kind of memory. Inactivation of the perirhinal area in rats led to deficits in object recognition in all memory processes, such as acquisition, consolidation and retrieval, although impairment in consolidation only occurred when the injuries were made within 20 minutes after acquisition [28]. A study evaluating object recognition memory in early HE patients described impairment for both STM

and LTM, however, it seems that the memory alterations observed were a consequence of attention and visual perception impairments rather than object recognition memory [29] (Weissenborn et al., 2003). In accordance to this assumption, a decrease in glucose uptake in frontal and parieto-occipital cortex areas but not in the temporal lobe was observed in patients with minimum HE [29,30]. Several studies have been performed in rat models of HE to evaluate visual perception and visual orientation, which appears to be harmed during HE [29,31]. Considering these findings, the possibility of an impaired visual perception in the BDL rats can not be discarded, since this would consequently interfere with object recognition memory. Moreover, visual and spatial perception deficits would also interfere with the locomotor and exploratory activity, which was observed for the BDL rats in the open field task.

In conclusion, the BDL rats exhibited altered spatio-temporal organization of locomotor and exploratory activities when facing the open field task and these behavioral alterations were not a consequence of motor deficits and anxiety behavior. In addition, an impairment of STM was observed for object recognition memory. The mechanisms leading to the impairment of the spatio-temporal locomotor and exploratory activities and object recognition memory are still not completely understood and further studies are fundamental to elucidate which brain areas and neurochemical mechanisms are involved.

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Table

Table 1: Serum Biochemical Mesuraments

Paramenters	SHAM	BDL
AAT (U/L)	37.83 ± 2.48	93.67 ± 9.19*
Alkaline Phosphatase (U/L)	65.83 ± 3.76	340.83 ± 24.41*
Direct Bilirubin (mg/dL)	0.01 ± 0.00	6.43 ± 0.28*
Total Bilirubin (mg/dL)	0.06 ± 0.01	10.54 ± 0.32*
Albumin (g/dL)	4.45 ± 0.09	2.08 ± 0.16*

Note: Data are represented as mean ± SEM. An asterisk (*) represents statistical significances ($p < 0.01$) by Student T test.

Figure Legends

Fig.1: Liver histopathology of the control (A and C) and BDL rats (B and D) liver. Representative liver sections (original magnification, x200) stained with H&E (A-B) and picrosirius (C-D). Note the increased bile duct proliferation (B) and collagen deposits (D) in the samples from the BDL rats.

Fig. 2: Locomotor and exploratory behavior in the open field task. Control (n=6) and BDL (n=5) rats were evaluated for: (A) total distance traveled, (B) time mobile/immobile, (C) number of stops, (D) inter-stops distance traveled, (E) number of rearing and (F) number of grooming. Data are represented as mean ± SEM. An asterisk (*) represents statistical significances ($p < 0.05$ by Student T test).

Fig. 3: Temporal organization of behavior in the open field task. Control (n=6) and BDL (n=5) rats were evaluated for: (A) time spent in the home base area, (B) number of trips, (C) trips length and (D) number of stops per trip. Data are represented as mean ± SEM. An asterisk (*) represents statistical significances ($p < 0.05$ by Student T test).

Fig. 4: Spatial distribution of behavior in the open field task. Control (n=6) and BDL (n=5) rats were evaluated for: (A) percentage of total distance traveled in the center zone and (B) percentage of time spent in the center zone. Data are represented as mean ± SEM. An asterisk (*) represents statistical significances ($p < 0.05$ by Student T test).

Fig. 5: Behavioral pattern in the plus maze task. Control (n=7) and BDL (n=5) rats were evaluated for: (A) number of entries on open (white bars) and closed (striped bars) arms and (B) time spent in the open (white bars) and closed (striped bars) arms. Data are represented as mean ± SEM. An asterisk (*) represents statistical significances ($p < 0.05$ by Two-way ANOVA followed by Bonferroni post hoc test).

Fig. 6: Number of foot faults performed by the control (n=8) and BDL (n=5) rats. Data are represented as mean ± SEM. There is no statistical difference between the groups.

Fig. 7: Object recognition memory. Control (n=7) and BDL (n=4) rats were evaluated for discrimination index (%) between: (A) objects A and B during training session; (B) objects A and C during STM, (C) objects A and D during LTM. Data are represented as mean ± SEM. An asterisk (*) represents statistical significances ($p < 0.05$ by Two-way ANOVA followed by Bonferroni post hoc test).

Fig. 8: (A) Schematic representation of spatio-temporal organization of locomotor and exploratory behavior for the control and BDL rats in the open field task. (B) Occupancy plot of the open field is represented as time (s) spent in the arena for control and BDL groups.

Fig.1 Supplement: Locomotor and exploratory behavior in the open field task. Control (n=6) and BDL (n=5) rats were evaluated for: (A) distance traveled across time, (B) time mobile across time, (C) number of rearing across time. Data are represented as mean ± SEM. An asterisk (*) represents statistical significances ($p < 0.05$ by Student T test).

Fig.1

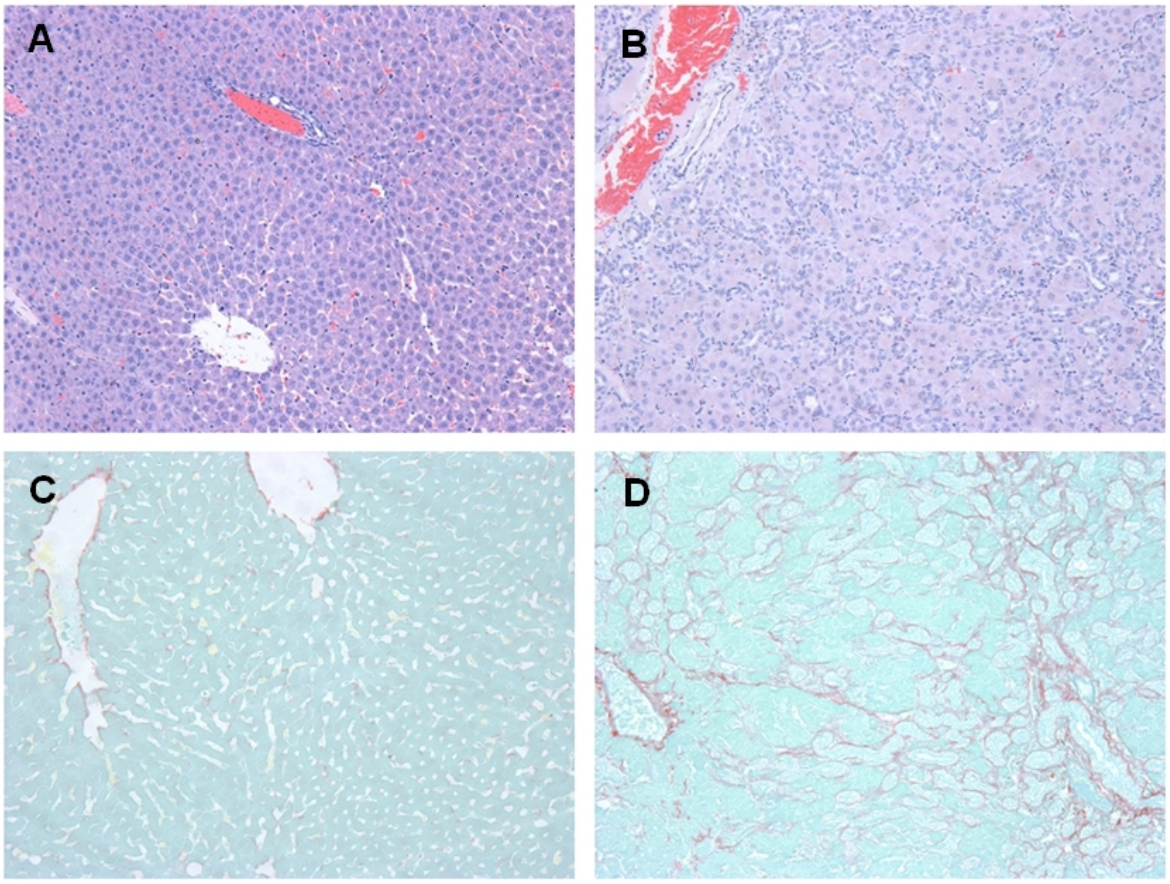

Fig. 2

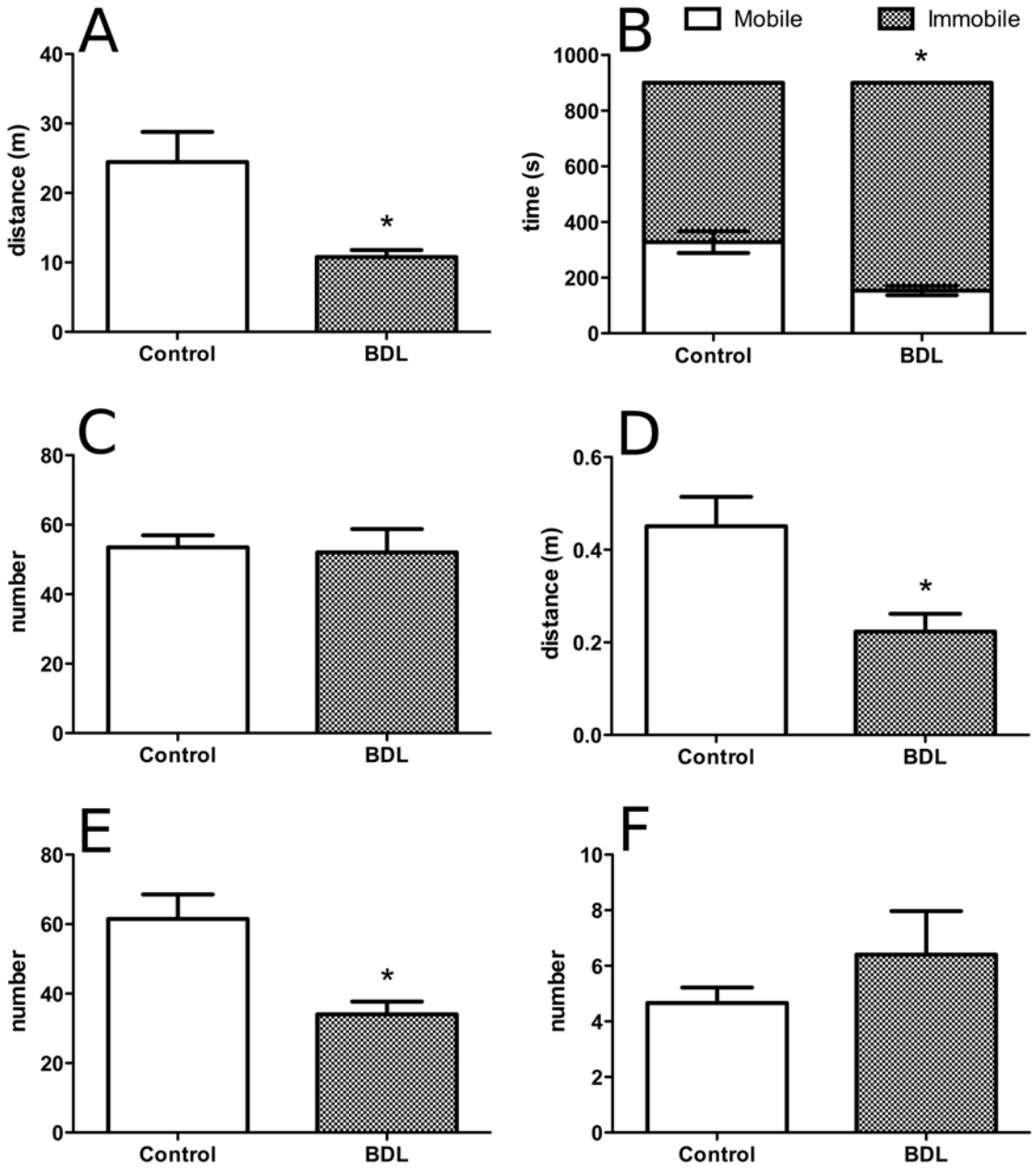
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Fig. 3

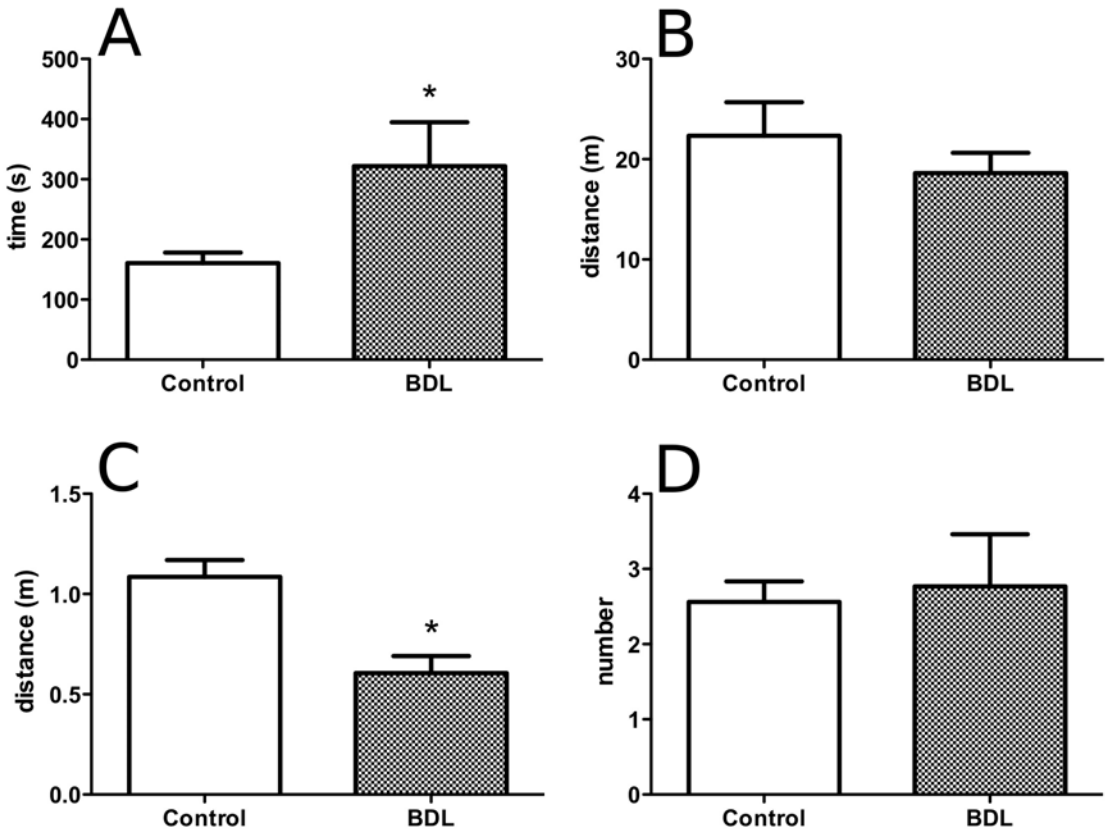
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Fig. 4

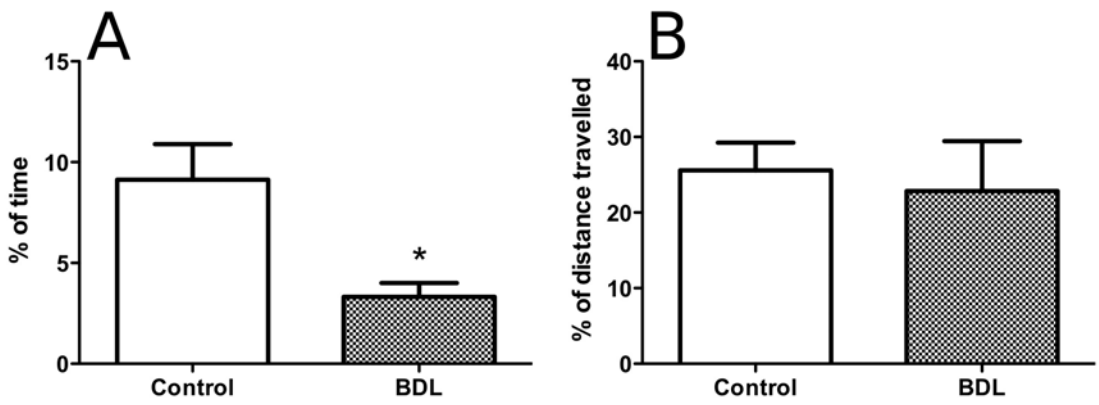
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Fig. 5

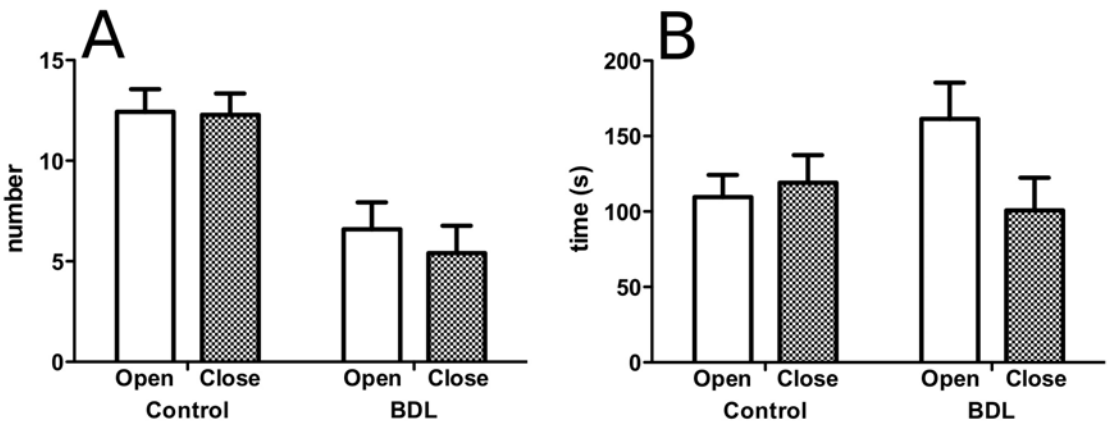

A
number
15
10
5
0
Open
Close
Open
Close
Control
BDL
B
time (s)
200
150
100
50
0
Open
Close
Open
Close
Control
BDL

Fig. 6

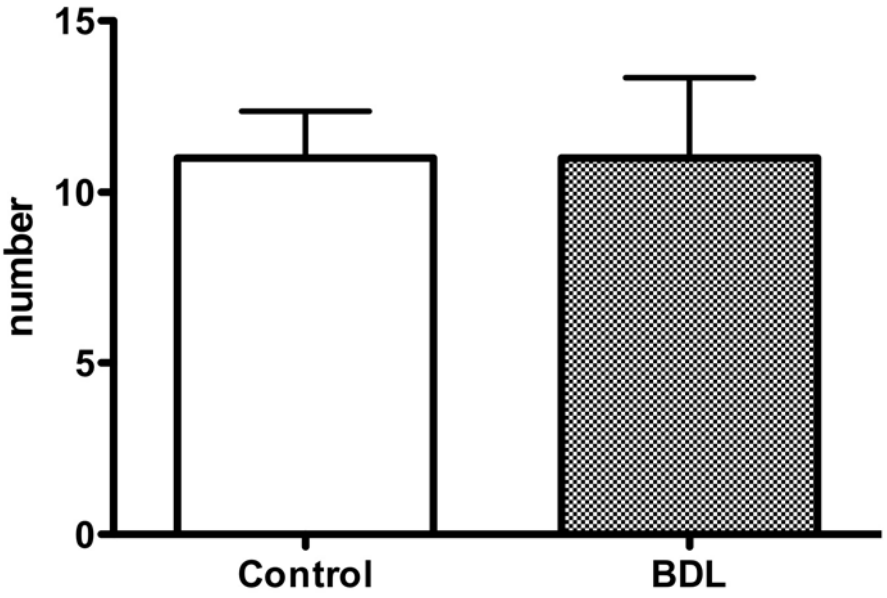


Fig. 7

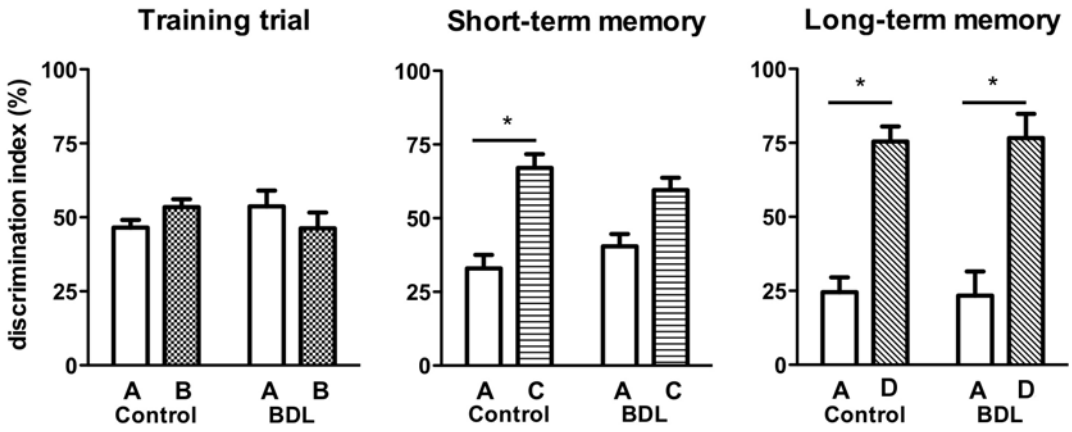

Training trial
Short-term memory
Long-term memory
discrimination index (%)
100
75
50
25
0
*
A B
Control
A B
BDL
A C
Control
A C
BDL
A D
Control
A D
BDL

Fig. 8

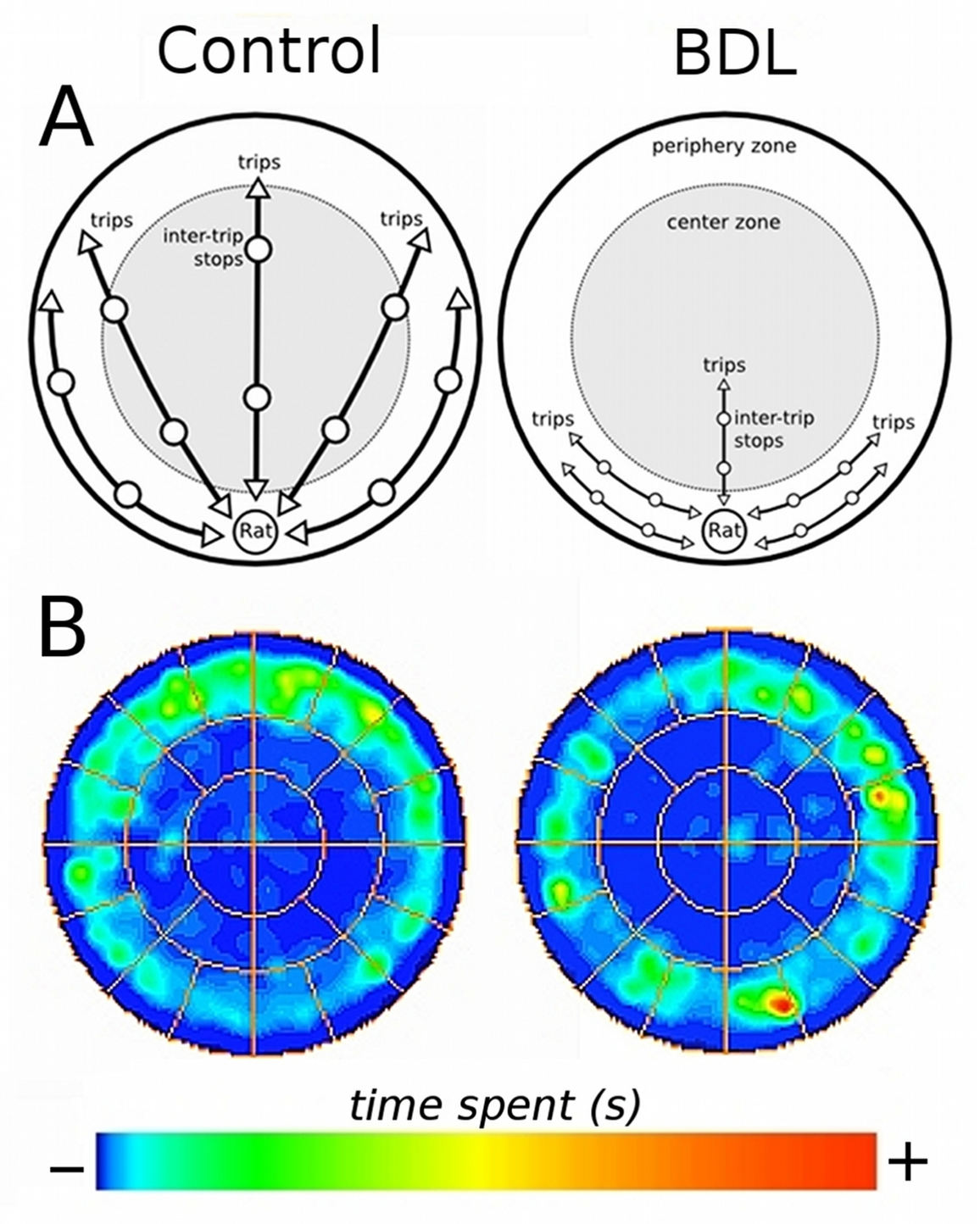
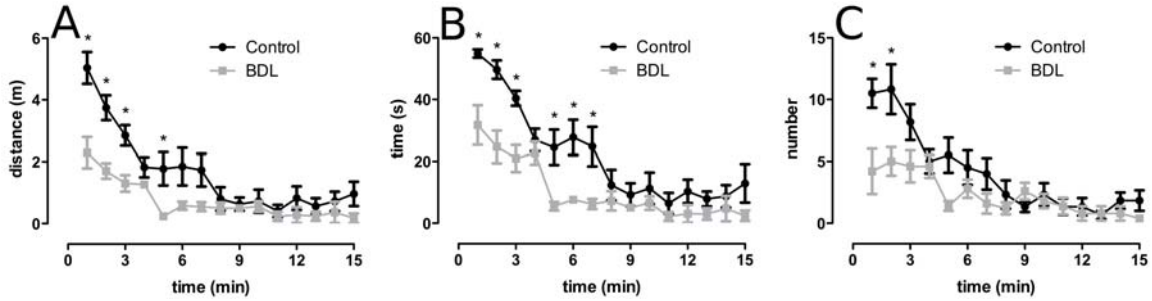

Control
BDL
A
trips
trips
trips
inter-trip
stops
Rat
periphery zone
center zone
trips
trips
inter-trip
stops
trips
Rat
B
time spent (s)
–
+

Fig. 1 Supplement



Parte III

3. Discussão

A HE é uma desordem neuropsiquiátrica que acomete uma grande parte dos pacientes que sofrem de falência aguda e crônica do fígado. Esta patologia que afeta o SNC pode ser manifestada clinicamente de diferentes formas, dependendo da etiologia da doença hepática e a velocidade em que esta última se desenvolve. Os mecanismos pelos quais a HE desenvolve-se ainda não estão completamente elucidados, mas muitos estudos têm sido realizados pela comunidade científica no intuito de melhor entende-los. Até o presente momento, diferentes mecanismos fisiopatológicos foram propostos e muitos estudos demonstram que o aumento da amônia na circulação sanguínea, e consequentemente sua ação no SNC, seja uma das principais causas do desenvolvimento da HE. Além disso, alterações no sistema neurotransmissor GABAérgico têm sido relacionadas com o desenvolvimento e/ou consequências patológicas da HE. Acredita-se que a neuroinibição causada pela ativação do sistema GABAérgico pode estar exacerbada durante a HE, e vários mecanismos foram propostos na tentativa de entender como isto ocorre, inclusive foi descrito o efeito direto e indireto da amônia (Bender and Norenberg, 2000; Felipo and Butterworth, 2002; Ahboucha and Butterworth, 2004).

Diversos modelos experimentais *in vitro* e *in vivo* são utilizados no meio científico para o estudo da HE. Os modelos *in vitro* normalmente utilizam culturas de diferentes tipos celulares, como astrócitos e neurônios. Muita ênfase tem sido dada no estudo dos efeitos da amônia sobre culturas de astrócitos, tendo em vista que este tipo celular é considerado o principal alvo celular da HE (Norenberg, 1998).

Desta forma, o primeiro objetivo desta tese (capítulo I) foi padronizar um método de co-cultura de neurônios e astrócitos GABAérgicos de fácil execução e que

refletisse a interação metabólica entre neurônios e astrócitos. O método de co-cultura de neurônios e astrócitos mais amplamente utilizado no meio científico é aquele onde primeiramente se cultivam astrócitos até que estes se tornem confluentes, para então haver o cultivo de neurônios sobre esta camada de astrócitos, formando então uma co-cultura. Esta técnica, apesar de ser bastante reproduzível, requer mais trabalho por parte de quem a executa, como também um maior período de tempo até que esteja pronta para ser utilizada. Por tais motivos, nós desenvolvemos um método de cultura onde uma suspensão de células, obtidas de córtex de fetos de camundongos era cultivada, originando uma cultura celular onde ambos os neurônios e astrócitos GABAérgicos estavam presentes. Para tal, o método de cultura descrito por Hertz e colaboradores (1989) foi utilizado, com exceção de que o agente citostático, citosina arabinose, não foi adicionado à cultura celular. Como consequência os astrócitos se proliferaram a ponto de formar a monocamada onde a interação neurônio astrócito foi estabelecida. No intuito de estudar a interação metabólica entre os neurônios e astrócitos GABAérgicos, uma série de experimentos foram realizados empregando a técnica de cromatografia líquida acoplada a um espectrômetro de massa (LC-MS), a cromatografia líquida de alta resolução (HPLC) e o uso de isótopos de carbono (^{13}C) como os substratos [U-^{13}C]acetato, [U-^{13}C]glicose, [U-^{13}C]lactato e [U-^{13}C]glutamina.

Os resultados realizados neste trabalho claramente demonstraram que neste método de cultura os neurônios e astrócitos interagem metabolicamente. Isto foi observado quando o substrato [U-^{13}C]acetato foi utilizado no meio de incubação, dando origem ao GABA contendo ^{13}C no extrato de células, pois somente os astrócitos têm a capacidade de metabolizá-lo pela enzyma acetil-CoA sintetase (Waniewski and Martin, 1998). O acetato foi metabolizado à glutamina e este composto foi subsequentemente

transferido do compartimento astrocitário para o neuronal, servindo de substrato para a biossíntese de GABA. Além disso, foi demonstrado que os astrócitos têm grande capacidade em metabolizar glutamina, visto através da razão da percentagem de incorporação de ^{13}C proveniente do acetato marcado. Os substratos marcados glicose e lactato também foram bons precursores para a marcação dos aminoácidos estudados.

A utilização do precursor [U-^{13}C]glutamina em diferentes concentrações também levou a importantes constatações. A primeira foi que a captação da glutamina exógena foi concentração dependente, chegando a um ponto de saturação, e foi preferencialmente metabolizada no compartimento neuronal. Também a incorporação de ^{13}C da glutamina nos aminoácidos glutamato, GABA e aspartato foi dependente das concentrações do substrato marcado. Estudos anteriores demonstraram que a síntese de GABA no compartimento neuronal a partir da glutamina envolve o ciclo de Krebs (Waagepetersen et al., 1999), achado que foi confirmado neste sistema de co-cultura de neurônios e astrócitos GABAérgicos. Além disso, analisando a razão entre a síntese de GABA diretamente da descarboxilação do glutamato, sem o envolvimento do ciclo de Krebs (chamada de síntese direta), pela síntese que envolve a metabolização do glutamato pelo ciclo de Krebs e subsequente descarboxilação (chamada de síntese via o ciclo de Krebs), demonstrou-se que esta tinha o valor aproximado de 1. Logo, a síntese de GABA ocorre igualmente pelas vias direta e ciclo de Krebs. Além disso, o aumento das concentrações exógenas de glutamina não alterou os valores da razão de síntese para GABA, porém aumentou para glutamato. Ou seja, o aumento de glutamina leva ao aumento da síntese direta de glutamato, mas não altera a de GABA. Estes dados demonstram que a existência de um "pool" de glutamato compartimetalizado.

Uma vez estando o modelo de co-cultura de neurônios e astrócitos GABAérgicos padronizado, o segundo objetivo desta tese (capitulo II) foi investigar o efeito da alta concentração de amônia sobre os processos de detoxificação celular cerebral e o metabolismo energético do sistema neurotransmissor GABAérgico. Neste trabalho, além da utilização do modelo de co-cultura de neurônios e astrócitos, também foi utilizado o sistema de culturas de neurônios GABAérgicos. Também foram utilizadas as técnicas de HPLC e LCMS, como também a tecnologia de ressonância nuclear magnética (NMRS). Para a realização dos meios de incubação foram usados os substratos marcados [U-^{13}C]glicose, [1,6-^{13}C]glicose, [U-^{13}C]lactato, β-[U-^{13}C]hydroxybutirate (BHB) e $^{15}NH_4Cl$.

O processo de detoxificação da amônia no cérebro ocorre principalmente através da atividade da enzima GS, que encontra-se localizada exclusivamente nos astrócitos (Cooper and Plum, 1987). Nossos resultados demonstraram que as co-culturas quando incubadas com amônia (aqui representada por $^{15}NH_4^+$) apresentaram uma alta percentagem de dupla marcação na glutamina. Isto indica que tanto o grupamento amina como amida estavam marcados, o que demonstra a alta atividade de ambas GDH e GS nos astrócitos. A glutamina é um bom agente detoxificante do cérebro, pois tem a capacidade de ser transportada para fora do SNC (Hawkins et al., 2006). Porém, para sustentar o aumento da síntese de glutamina, é necessário a síntese *de novo* de α-cetoglutarato a partir da atividade da enzima piruvato carboxilase (PC), para o fornecimento de unidades de carbono. O aumento da atividade da PC, em relação à atividade da enzima PDH, também foi verificado nas co-culturas GABAérgicas incubadas com amônia. Logo, o aumento da síntese de glutamina estava sendo sustentada pelo aumento da atividade da rota anaplerótica e subsequente síntese

de α-cetoglutarato. A atividade da enzima GDH também parece ser importante para detoxificar o excesso de amônia. Neste trabalho nós demonstramos a alanina e o aspartato estavam marcados em aproximadamente 40% a partir da amônia (aqui representada por $^{15}NH_4^+$). Logo, no sistema de co-cultura GABAérgica, a atividade da GDH é bastante importante porque a amônia livre é unida ao α-cetoglutarato, formando glutamato que subsequentemente sofre reações de transaminação para a formação de alanina e aspartato. A síntese de alanina também pode funcionar como um processo detoxificante de amônia. Estudos realizados com culturas celulares e modelos animais demonstraram o aumento da síntese de alanina durante a hiperamonemia (Hindfelt et al., 1977; Zwingmann et al., 2003; Zwingmann and Leibfritz, 2005). Nós demonstramos que durante a exposição à amônia a síntese de alanina, a partir da glicose aumentou, como também e a sua concentração extracelular. Estes resultados mostram que a alanina estava sendo liberada para o meio extracelular. Sabe-se que a alanina apresenta um sistema de transporte na barreira hematoencefálica e por isso podemos sugerir que o aumento da síntese e liberação de alanina possa ser um mecanismo de detoxificação, uma vez que este aminoácido pode ser transportado para fora do SNC (Hawkins et al., 2006). É importante salientar que a síntese de alanina é a única forma de detoxificação de amônia dos neurônios, uma vez que este tipo celular não tem a enzima GS e PC (Norenberg and Martinez-Hernandez, 1979; Waagepetersen et al., 2002; Lovat et al., 2007)

Tem sido descrita a inibição do ciclo de Krebs durante a HE e hiperamonemia, principalmente devido à inibição das enzimas PDH e α -cetoglutarato desidrogenase.Ao contrário do que têm sido proposto nós demonstramos que no modelo de co-cultura de neurônios e astrócitos GABAérgicos o ciclo de Krebs têm a sua atividade aumentada

após a incubação com amônia. Tanto a co-culturas como a cultura de neurônios apresentaram o aumento da incorporação de ^{13}C da [U-^{13}C]glicose nos aminoácidos estudados. Além disso, através da marcação de ^{13}C proveniente da [1,6-^{13}C]glicose (analisado por NMRS), foi determinado o aumento da razão de voltas do ciclo de Krebs nos neurônios ("cycling ratio" para GABA). Porém, este aumento não foi observado nos astrócitos ("cycling ratio" para glutamina), possivelmente porque o α-cetoglutarato estava sendo deslocado do ciclo de Krebs para a síntese de glutamato e glutamina. Quando o [U-^{13}C]lactato foi utilizado como substrato também foi demonstrado o aumento da incorporação de ^{13}C nos aminoácidos glutamato, GABA e aspartato. Porém, a percentagem de incorporação para a glutamina diminuiu e isto pode ter ocorrido porque o lactato pode não ser um bom substrato para sustentar as células do sistema de cultura energeticamente. O substrato BHB foi empregado neste trabalho somente como uma ferramenta para a avaliação do ciclo de Krebs sem o envolvimento da glicólise e metabolismo do lactato. As culturas de neurônios quando incubadas com BHB e amônia mostraram o aumento de incorporação de ^{13}C no glutamato, o que confirma o aumento da atividade do ciclo de Krebs. É importante salientar que os níveis de ATP diminuíram muito quando o BHB foi utilizado como único substrato, demonstrando que este substrato sozinho não é capaz de manter as células energeticamente. Possivelmente, os aminoácidos foram utilizados como substrato energético, levando à diminuição da incorporação de ^{13}C.

Neste estudo nós também demonstramos que a amônia leva ao aumento da glicólise em ambos os sistemas de cultura estudados, que foi detectado pelo aumento da incorporação de ^{13}C na alanina proveniente da glicose. De acordo com este achado, outros estudos haviam descrito o aumento da glicólise (Ratnakumari and Murthy, 1992;

Johansen et al., 2007). Além do aumento da alanina, também observamos o aumento das concentrações extracelulares de lactato, o que também indica o aumento da glicólise. É bastante interessante o fato de que quando o lactato foi utilizado como substrato energético, os níveis de ATP diminuíram drasticamente, na presença e ausência da amônia. Ao contrário, a glicose manteve os níveis de ATP normais tanto para as culturas controle como para as incubadas com amônia. A diminuição observada nos níveis de ATP quando incubadas com lactato pode ser devido ao aumento da razão de NADH/NAD+, que pode inibir a glicólise e afetar o "shutle" de malato e aspartato.

Desta forma, o trabalho desenvolvido no capítulo II demonstrou que a alanina é um potencial agente detoxificante de amônia em neurônios GABAérgicos. Além disso, demonstramos que a amônia estimula a atividade do ciclo de Krebs em neurônios GABAérgicos e também aumenta a glicólise em ambos os neurônios e astrócitos GABAérgicos. Além disso, demonstrou-se que a glicose é um substrato fundamental para a sustentação energética das células presentes na cultura celular.

Considerando que nós observamos profundas alterações no metabolismo energético e nos processos de detoxificação de amônia no sistema de co-cultura GABAérgica, possivelmente a síntese do GABA também pode estar sendo afetada durante a HE e hiperamonemia. Sendo assim, o terceiro objetivo desta tese (capítulo III), foi estudar os efeitos da HE e hiperamonemia sobre a síntese de GABA tanto no modelo de co-culturas GABAérgicas, como também no modelo *in vivo* de HE, induzido pela ligação do ducto biliar (BDL), em ratos. Assim, o substrato [U-^{13}C]acetato foi utilizado tanto no modelo *in vivo* como *in vitr*o, e a [U-^{13}C]glutamina foi empregada nos estudos *in vitro*.

Neste trabalho nós demonstramos que a síntese de GABA ocorreu preferencialmente *via* o ciclo de Krebs tanto nos ratos BDL como nas co-culturas de neurônios e astrócitos GABAérgicos incubados com amônia. Mais especificamente, os ratos BDL apresentaram o aumento do GABA marcado apenas com um carbono 13, que denota a sua síntese via ciclo de Krebs. Já as culturas de neurônios e astrócitos GABAérgicos apresentaram a diminuição do GABA duplamente marcado, que ocorre na síntese diretamente do glutamato, sem o envolvimento do ciclo de Krebs. Mesmo não apresentando o mesmo padrão de marcação, o resultado final é que em ambos os modelos a síntese de GABA via o ciclo de Krebs está favorecida durante a HE e hiperamonemia. O aumento da síntese de GABA por esta via pode ser bastante significativo, pois a síntese de GABA via o ciclo de Krebs está relacionada com o "pool" vesicular deste neurotransmissor, ou seja, o GABA que será posteriormente responsável pela neurotransmissão GABAérgica.

Um dos fatores que poderia estar favorecendo a síntese de GABA através do ciclo de Krebs seria o aumento das concentrações de glutamina, induzida pela ação tóxica da amônia. No intuito de elucidar este possível mecanismo, as co-culturas de neurônios e astrócitos GABAérgicos foram incubadas com três diferentes concentrações de glutamina (0,1, 0,3 e 0,5 mM), juntamente com a amônia. Os resultados obtidos neste estudo mostraram que a amônia levou à diminuição da incorporação (molecular carbon labeling - MCL) de ^{13}C da glutamina nos aminoácidos glutamato, GABA, aspartato e glutamina. Isto pode ter acontecido possivelmente pelo aumento da síntese de glutamina não marcada pelos astrócitos, levando à diluição da glutamina marcada e afetando a marcação dos aminoácidos. Como demonstramos no trabalho anterior, a amônia induz o aumento da glicólise e atividade da PC, que levam

ao aumento da síntese de glutamina. Quando analisamos a razão entre a síntese de GABA direta pela síntese via o ciclo de Krebs a partir da glutamina, verificamos que a amônia não influenciou a via de síntese do GABA. Logo, o aumento das concentrações exógenas de glutamina, juntamente com a presença da amônia, não alteram por qual rota o GABA é sintetizado. Uma vez estando esta hipótese descartada, acreditamos que o favorecimento da síntese de GABA via o ciclo de Krebs possa ser uma consequência do aumento da atividade deste último. Porém, este mecanismo pode não ser a única causa dos efeitos observados. Temos que levar em consideração que a amônia possa influenciar diferentes enzimas responsáveis pela síntese de GABA. Sendo assim, mais estudos são necessários para elucidar a importância do aumento da síntese de GABA via o ciclo de Krebs, como também outros possíveis mecanismos relacionados com este achado.

Os resultados que foram discutidos até agora claramente demonstram que a ambos os neurônios GABAérgicos e astrócitos têm a sua atividade alterada durante a hiperamonemia. Além disso, no último capítulo, também verificamos que a síntese de GABA estava alterada no modelo de HE em ratos. Considerando estes resultados, como também a descrição das manifestações clínicas de hipolocomoção e prejuízos cognitivos descritos em humanos e animais (Weissenborn et al., 2005a; Cauli et al., 2007), no capítulo IV procuramos estudar os efeitos da HE sobre parâmetros comportamentais e cognitivos em ratos. Para verificar a atividade locomotora e exploratória dos ratos BDL o aparato de campo aberto foi utilizado.Também, o teste de reconhecimento de objeto foi empregado para avaliar a memória de reconhecimento destes animais.

Os resultados obtidos neste trabalho mostram que os ratos BDL apresentam a diminuição da atividade locomotora e exploratória, quando comparados com os animais controle. Além disso, eles apresentam uma alteração na organização espacial e temporal da atividade exploratória e locomotora. Porém, os prejuízos observados nesta tarefa não são uma consequência de problemas motores e de ansiedade, uma vez que os ratos BDL não diferiram dos controles nas tarefas de "foot fault" e labirinto em cruz elevado (plus maze). Diferentes trabalhos descrevem a diminuição da locomoção em modelos animais de HE (Cauli et al., 2007; Ahboucha et al., 2008). Porém estes trabalhos normalmente avaliam somente a atividade locomotora através da distancia total percorrida, número de crossing e rearing, e não exploram outras informações sobre a organização espacial e temporal da atividade locomotora e exploratória. Em relação aos possíveis mecanismos envolvidos na diminuição das atividades locomotora e exploratória, têm sido proposta a disfunção dos ganglios da base, como também das conexões entre os ganglios da base, tálamo e córtex motor (Jover et al., 2005; Giewekemeyer et al., 2007). Também foi demonstrada que a alteração do funcionamento glutamatérgico e GABAérgico pode estar relacionada com a hipolocomoção (Ahboucha et al., 2008).

Na tarefa de reconhecimento de objeto, os ratos BDL demonstraram o prejuízo na memória de curta duração, porém não na de longa duração. Ao contrário do que observamos, o estudo realizado por Gárccia moreno (2005) demonstrou a diminuição da memória de longa duração em ratos BDL. Porém, devemos levar em consideração que o protocolo empregado por estes autores é muito diferente do que utilizamos. De acordo com os resultados obtidos nos ratos BDL, foi descrito o prejuízo da memória de reconhecimento, tanto a memória de curta como a de longa duração, em pacientes com

HE (Weissenborn et al., 2003). Porém, este resultado foi interpretado como uma possível disfunção na atenção e prejuízos da percepção visual. Diferentes trabalhos já relatam problemas que ratos com HE apresentam problemas de percepção visual e espacial. Logo, não podemos descartar a possibilidade de que os ratos BDL estejam com problemas de percepção espacial e visual, o que consequentemente afetaria tanto os testes de reconhecimento de objeto como também as atividades de locomoção e exploração.

Desta forma, os resultados obtidos neste trabalho são bastante interessantes pois demonstram que os ratos BDL têm alterada a organização espaço-temporal das atividades locomotora e exploratória. Também, os ratos BDL têm prejuízos na memória de curta duração de reconhecimento de objeto. Para o melhor entendimento dos prejuízos locomotores e cognitivos aqui descritos o desenvolvimento de outros estudos são necessários na tentativa de desvendar os possíveis mecanismos neuroquímicos e as regiões cerebrais que estão envolvidas nestes processos deletérios observados durante a HE.

4. Conclusão

Nesta tese um modelo de co-cultura de neurônios GABAérgicos e astrócitos foi padronizado, demonstrando-se bastante reproduzível, de fácil execução e representativo da interação metabólica entre neurônios e astrócitos. Também, foi verificado que a amônia, considerada um dos principais fatores associados ao desenvolvimento da HE, altera o metabolismo energético do sistema neurotransmissor GABAérgico, aumentando a glicólise e a atividade do ciclo de Krebs. A síntese e liberação de alanina também encontrou-se aumentada, e seu papel como um agente detoxificante de amônia pode ser fundamental para a sobrevivência dos neurônios GABAérgicos.

A síntese de GABA mostrou-se alterada tanto em ratos BDL como também nas co-culturas de neurônios GABAérgicos e astrócitos expostos à amônia. Detectou-se que em ambos os modelos a síntese de GABA relacionada com o ciclo de Krebs estava favorecida. Também, empregando o modelo BDL, constatamos que estes animais com HE apresentavam prejuízos nas atividades locomotora e exploratória, como também na memória de curta duração para o reconhecimento de objeto.

De uma maneira geral, esta tese descreve novos achados neuroquímicos e comportamentais que podem ser importantes para o entendimento dos mecanismos patofisiológicos da HE, como também reforça certos achados descritos na literatura científica que afirmam a existência da alteração no sistema de neurotransmissão GABAérgico. A amônia, que aqui foi utilizada para os estudos *in vitro*, estando também aumentada no plasma dos ratos BDL, é de fato um agente neurotóxico que exerce papel fundamental nos mecanismos deletérios observados durante a HE.

5. Perspectivas

Temos o objetivo de dar continuidade aos estudos aqui apresentados, buscando elucidar as novas perguntas que emergiram durante o desenvolvimento desta tese. Desta forma, as perspectivas são:

- Avaliar pela técnica de Western Blot a expressão dos transportadores de glutamina nas co-culturas de neurônios GABAérgicos e astrócitos.

- Aprofundar os estudos sobre a importância do papel da alanina nos processos de detoxificação de amônia no modelo BDL em ratos.

- Avaliar a atividade da enzima GAD em córtex e hipocampo de cérebro de ratos BDL, assim como verificar por real time-PCR e Western Blot a transcrição e expressão das isoformas da enzima GAD, GAD_{65} e GAD_{67}.

- Estudar através de experimentos comportamentais a percepção visual e espacial de ratos BDL através das atividades comportamentais "Y-maze" e "water maze".

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