

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

**ALTERAÇÕES BIOQUÍMICAS, HISTOLÓGICAS E COMPORTAMENTAIS EM
RATOS SUBMETIDOS À ADMINISTRAÇÃO INTRACEREBROVENTRICULAR
DE GALACTOSE**

ANDRÉ FELIPE RODRIGUES

Orientadora: Prof^ª. Dr^ª. ANGELA TEREZINHA DE SOUZA WYSE

PORTO ALEGRE

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Este trabalho é dedicado a todos os que compõem minha família

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Don't stop doing what you believe in

Donavon Frankenreiter

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RESUMO

Altos níveis de galactose circulantes e cerebrais são encontrados em portadores da galactosemia clássica não tratada, cujos pacientes comumente desenvolvem problemas cognitivos e motores ao longo da vida. Entretanto pouco se conhece a respeito dos mecanismos da disfunção celular e molecular responsáveis por estes sintomas. Assim, o objetivo do presente trabalho foi de investigar o efeito da injeção intracerebroventricular de galactose sobre a memória (aversiva e de reconhecimento de objetos) e a coordenação motora em ratos Wistar. Além disso, a atividade, o imunoconteúdo e a expressão gênica da acetilcolinesterase no hipocampo e córtex cerebral foram também avaliados. No cerebelo, foram medidos parâmetros histológicos (contagem de células e imunohistoquímica) e bioquímicos (imunoconteúdo de caspase-3 ativa e BDNF, atividade e imunoconteúdo da acetilcolinesterase, níveis de glutatona e sulfidrilas, bem como o índice de dano ao DNA). Ratos Wistar receberam uma injeção intracerebroventricular de galactose (4 mM) ou salina (controles) sendo esses submetidos às tarefas comportamentais e/ou decapitados em diferentes tempos (1 h, 3 h ou 24 h), logo após, o hipocampo, córtex cerebral e cerebelo foram dissecados. Os resultados mostraram que a galactose prejudicou a memória aversiva e de reconhecimento de objetos, quando injetada antes do treinamento, bem como alterou a atividade e a expressão gênica da acetilcolinesterase em hipocampo. Em relação ao comportamento motor e aos parâmetros histológicos e bioquímicos realizados no cerebelo, a administração intracerebroventricular de galactose prejudicou a coordenação motora e reduziu o número de células e a imunomarcagem de neurônios e astrócitos. A galactose, também aumentou o imunoconteúdo de caspase-3 ativa, a atividade da acetilcolinesterase e o índice de dano ao DNA, bem como diminuiu o imunoconteúdo de BDNF e acetilcolinesterase e os níveis de glutatona e sulfidrilas no cerebelo. Tomados em conjunto, nossos resultados mostram que a administração intracerebroventricular de galactose prejudicou a memória e a coordenação motora. Além disso, o modelo experimental utilizado mostrou diversas alterações a nível celular e molecular, os quais podem contribuir pelo menos em parte com o entendimento da fisiopatologia da galactosemia clássica.

Palavras-chave: galactosemia clássica; memória; coordenação motora; acetilcolinesterase; apoptose

ABSTRACT

Non-treated patients with classical galactosemia present high levels of galactose in the bloodstream and brain. Patients usually develop cognitive and motor impairments during life. However, little is known about the cellular and molecular mechanisms responsible for these symptoms. Thus, the aim of this study was to investigate the effect of intracerebroventricular galactose injection on memory (aversive and object recognition) and motor coordination in Wistar rats. Acetylcholinesterase activity, immunocontent and gene expression were investigated in hippocampus and cerebral cortex. In the cerebellum, we performed histological (cell counting and immunohistochemistry) and biochemical (active caspase-3 immunocontent, BDNF, acetylcholinesterase activity and immunocontent, glutathione and sulfhydryl levels, as well as DNA damage index) parameters. Wistar rats received a single intracerebroventricular injection of galactose (4 mM) or saline (control). The animals performed behavioral tasks and/or were decapitated at different times (1 h, 3 h or 24 h) after injection. The hippocampus, cerebral cortex and cerebellum were dissected. The results showed that injecting galactose before training provokes impairment on aversive and object recognition memories, as well as altered the activity and gene expression of acetylcholinesterase in hippocampus. Regarding to the histological and biochemical parameters measured in the cerebellum, intracerebroventricular galactose injection impaired motor coordination, reduced the number of cells and immunostaining of neurons and astrocytes. In the cerebellum, galactose also increased active capase-3 immunocontent, acetylcholinesterase activity and DNA damage index, as well as decreased BDNF and acetylcholinesterase immunocontent, and glutathione and sulfhydryl levels. Altogether, our results show that intracerebroventricular injection of galactose impaired memory and motor coordination. Moreover, the experimental model used showed several alterations at cellular and molecular levels. These findings may contribute at least in part with the understanding of the physiopathology in classical galactosemia.

Key words: classical galactosemia; memory; motor coordination; acetylcholinesterase; apoptosis

LISTA DE ABREVIATURAS

AChE - acetilcolinesterase

BDNF - fator neurotrófico derivado do encéfalo

CD11b - receptor complementar 11b

EIM - erros inatos do metabolismo

GAL - galactose

GALE - UDP-galactose 4' epimerase

GALK - Galactokinase

GALT - galactose-1-P uridiltransferase

GC - galactosemia clássica

GFAP - proteína fibrilar acida glial

GSH - glutationa

H&E - hematoxilina e eosina

IL- 1 β - inteleucina 1 β

IL6 - inteleucina 6

NeuN - antígeno nuclear neurônio específico

PI - iodeto de propídeo

SNC - sistema nervoso central

TNF- α - fator de necrose tumoral

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1. INTRODUÇÃO

1.1. Erros inatos do metabolismo

Os erros inatos do metabolismo (EIM) foram definidos por Garrod em 1908 a partir de quatro doenças (albinismo, cistinúria, alcaptonúria e pentosúria). Os EIM são doenças genéticas, caracterizadas pela falta ou síntese anômala de uma determinada proteína, geralmente uma enzima ou um transportador (Olsen et al., 2015; Scriver, 2008). Essa interrupção metabólica pode levar ao acúmulo de metabólitos tóxicos e/ou falta de produtos essenciais (Mak et al., 2013; Olsen et al., 2015). A sintomatologia desenvolvida pode variar de leve à severa, dependendo do EIM em questão e do tecido afetado (Nasser et al., 2009). Tem sido mostrado que doenças que afetam o neurodesenvolvimento podem acarretar alterações comportamentais, cognitivas e/ou físicas. Neste contexto, um estudo realizado na China, que incluiu 285 pacientes com desordens de desenvolvimento do sistema nervoso central, mostrou que 36% das desordens estavam associadas a algum EIM (Guo et al., 2011).

Atualmente a literatura reconhece mais de 1000 diferentes tipos de EIM. Quando analisados individualmente, os EIM são considerados doenças raras, porém quando analisados em conjunto a incidência pode chegar a 1:800 nascidos vivos (Mak et al., 2013). É importante destacar que os EIM podem acometer qualquer grupo étnico (Mak et al., 2013), porém a frequência de determinado EIM pode variar em determinadas populações, especialmente devido ao fato de que

um quinto dos casais da população mundial é formado por matrimônios consanguíneos (Hamamy, 2012).

Os EIM são classificados de acordo com a área metabólica afetada, assim são encontrados EIM dos: carboidratos, aminoácidos, ácidos orgânicos, lipídeos, glicoproteínas entre outros (Scriver, 2001). Quanto aos EIM dos carboidratos, os mais comuns são os de armazenamento de glicogênio, do metabolismo da galactose (GAL) e do metabolismo da frutose (Mayatepek et al., 2010).

1.2. Metabolismo da galactose

A GAL é um monossacarídeo, obtido principalmente através do leite e seus derivados. A clivagem da lactose ocorre por ação enzimática da lactase nas microvilosidades intestinais da porção do jejuno, onde também ocorre a absorção dos monossacarídeos resultantes, glicose e GAL (Devaraj et al., 2013; Fridovich-Keil and Walter, 2008). A GAL é então transportada pelos eritrócitos até o fígado através da veia porta hepática, neste tecido a GAL é convertida em glicose (Dashty, 2013) (Figura 1).

A rota metabólica de conversão da GAL em glicose é também conhecida como via de Leloir, esta via é conservada evolutivamente desde bactérias até humanos. Três enzimas distintas compõem esta rota, no primeiro passo a α -D-GAL é fosforilada pela galactoquinase (GALK, EC 2.7.1.6), formando galactose-1-P, posteriormente, a enzima galactose-1-P uridiltransferase (GALT, EC 2.7.7.12), catalisa a reação entre galactose-1-P e UDP-glicose, neste processo uma UMP é transferida da UDP-glicose para galactose-1-P resultando na produção de glicose-

1-P e UDP-galactose. No último passo, a enzima UDP-galactose 4' epimerase (GALE, EC 5.1.3.2) interconverte UDP-galactose em UDP-glicose (Dashty, 2013; Fridovich-Keil and Walter, 2008; Slepak et al., 2005), como mostrado na Figura 2.

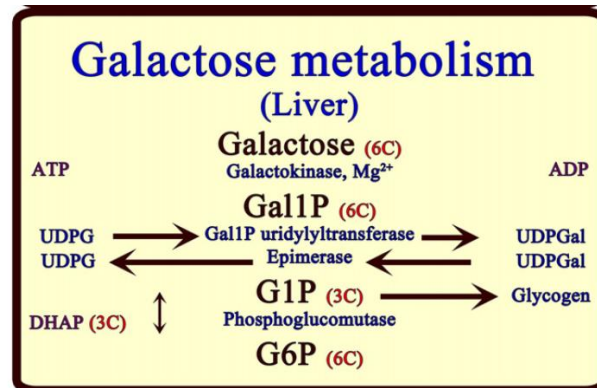


Figura 1 – Metabolismo hepático da galactose, Gal1P: galactose 1-P; G1P: glicose 1-P; G6P: glicose 6-P; DHAP: diidroxiacetona-P; 3C: 3 carbonos; 6C: 6 Carbonos. Adaptado de Dashty (2013)

A diminuição ou ausência da atividade de uma das três enzimas mencionadas acima bloqueia a rota da GAL causando três doenças distintas, conhecidas como galactosemias. O acúmulo de GAL e/ou metabólitos gerados, inicia um quadro de toxicidade celular que pode se estabelecer em diferentes tecidos, incluindo o cérebro (Lai et al., 2009; Slepak et al., 2005). A galactosemia clássica, é a forma mais severa dentre as galactosemias, é causada pela deficiência da enzima GALT (Fridovich-Keil and Walter, 2008).

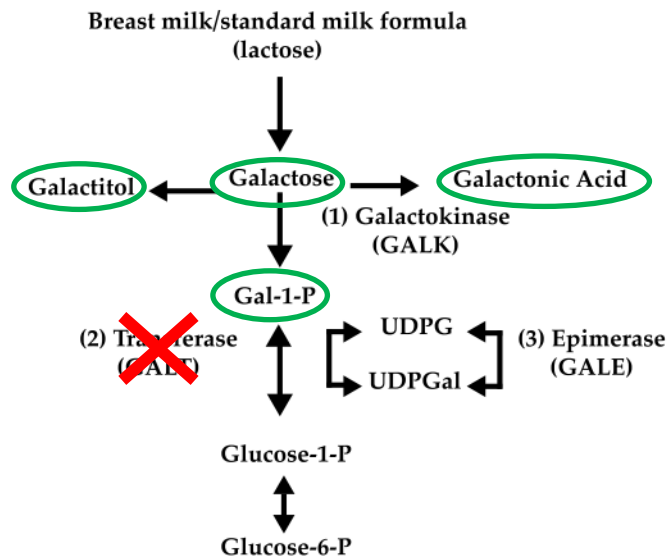


Figura 2 – Rota metabólica da galactose, o “X” em vermelho indica o bloqueio enzimático característico da galactosemia clássica, circulado em verde os substratos acumulados devido ao bloqueio enzimático. GALK: galactokinase; GALT: galactose 1-P uridiltransferase; GALE: UDP-galactose 4’ epimerase; Gal 1-P: galactose 1-P; UDPG: UDP- glicose; UDPGal: UDP-galactose. Adaptado de Dios *et al.*, (2009).

1.3. Galactosemia clássica (GC)

A GC também conhecida como galactosemia tipo I foi descrita em 1956 por Isselbacher e colaboradores (1956). A frequência baseada em números absolutos de nascidos vivos com GC varia consideravelmente de uma população para outra (Walter and Fridovich-Keil, 2014). Mundialmente a GC é apontada como o segundo EIM mais recorrente. Um estudo realizado no Canadá mostrou que a GC e a fenilcetonúria juntas, perfazem um quarto dos EIM (Applegarth et al., 2000). A África do Sul possui a maior incidência de GC, 1:14.400 (Henderson et al., 2002). Um projeto piloto realizado no estado de São Paulo mostrou que a frequência de

GC esta em torno de 1:19.984 neste local (Camelo Junior et al., 2011). Em algumas populações dos Estados Unidos e Canadá a incidência gira em torno de 1:35.000 (Fridovich-Keil and Walter, 2008). Por outro lado, a GC parece ser mais rara em populações asiáticas, com incidência de 1:100.000 (Padilla et al., 2003).

A GC é uma desordem de origem genética autossômica recessiva, ligada ao gene que codifica a enzima GALT, sendo este localizado no braço curto do cromossomo 9 na banda 13, este gene é dividido em 11 exons. Os exons 6, 9 e parte do 10 possuem uma sequência de aminoácidos altamente conservada, desde bactérias até humanos (Leslie et al., 1992; Shih et al., 1984).

Atualmente mais de 200 mutações foram identificadas no gene que codifica a GALT, estas acarretam em diminuição ou perda da sua atividade (Calderon et al., 2007; Fridovich-Keil and Walter, 2008). As mutações Q188R e K285N perfazem 70% dos casos de GC em populações caucasianas americanas e a mutação S135L afeta 62% de portadores afrodescendentes americanos (Elsas and Lai, 1998). Na mutação Q188R a glutamina da posição 188 é substituída por arginina (Reichardt et al., 1991), enquanto na mutação K285N, a lisina da posição 285 é trocada por uma glutamina (Greber-Platzer et al., 1997), no caso da mutação S135L, a serina da posição 135 é substituída por uma leucina (Lai et al., 1996).

1.3.1. Sinais e sintomas

Os sinais e sintomas característicos da GC podem ser divididos em agudos e crônicos. As principais complicações agudas descritas são: vômitos, diarreia, problemas renais, catarata, hepatomegalia e sepse. Caso a patologia não seja

diagnosticada rapidamente e/ou o tratamento não seja imediatamente iniciado o paciente pode vir a óbito nos primeiros dias de vida (Fridovich-Keil and Walter, 2008; Waggoner et al., 1990). Entretanto, mesmo com o tratamento recomendado, grande parte dos pacientes desenvolvem em estágios iniciais da vida, problemas cognitivos, dificuldades na fala, alterações motoras e falha precoce ovariana (Fridovich-Keil et al., 2011; Jumbo-Lucioni et al., 2013; Kaufman et al., 1995; Potter et al., 2013; Waggoner et al., 1990).

A deficiência na atividade da GALT leva ao acúmulo tecidual de GAL, galactose-1P e galactitol, a GAL é encontrada em maior concentração acumulada por ser o primeiro metabólito da rota (Fridovich-Keil and Walter, 2008; Schulpis et al., 2005). As concentrações cerebrais de GAL e galactose-1P em pacientes não tratados podem atingir concentrações na faixa de milimolar (Berry, 2011), podendo variar de acordo com a mutação e dieta (Fridovich-Keil and Walter, 2008). Mesmo com uma dieta restrita de GAL, foi demonstrado que indivíduos adultos portadores da GC ou não, com cerca de 70 Kg produzem diariamente uma média de dois gramas de GAL (Berry et al., 1995). Acredita-se que a produção endógena de GAL inicia ainda durante a vida intrauterina, pois elevados níveis de galactitol e galactose-1P foram encontrados em cordão umbilical, eritrócitos e líquido amniótico de recém-nascidos com GC (Berry, 2011).

Análises de ressonância magnética e tomografia computadorizada em portadores da doença, com sintomas neurológicos em estágio avançado da doença, apontam para anormalidades na substância branca cerebral (Fridovich-Keil and Walter, 2008), aumento ventricular e atrofia cerebelar e cortical (Kaufman et al., 1995). Redução da densidade óssea e atraso de crescimento são outras

consequências que podem ser desencadeadas em longo prazo (Fridovich-Keil and Walter, 2008).

1.3.2. Mecanismos de toxicidade

Diferentes mecanismos de toxicidade são apontados como mecanismos fisiopatológicos da GC (Lai et al., 2009). A galactose-1P é acumulada somente na GC. Este metabólito tem sido fortemente associado com a sintomatologia da GC (Fridovich-Keil and Walter, 2008; Gitzelmann, 1995; Schulpis et al., 2005). Esta hipótese é reforçada já que na galactosemia do tipo II, onde não há o acúmulo de galactose-1P a sintomatologia é branda, não sendo observada mortalidade (Fridovich-Keil and Walter, 2008).

Por outro lado, o acúmulo de galactitol pode alterar o metabolismo do inositol, desencadeando uma série de desordens a nível celular (Berry, 2011). Vale ressaltar que na galactosemia do tipo II, em que o galactitol é o principal metabólito acumulado, não há a manifestação dos sintomas cerebrais e em apenas alguns casos ocorre o desenvolvimento de edema cerebral. Nesse contexto, tem sido usado um modelo nocaute para GALT com o objetivo de investigar a sintomatologia da GC. No entanto esse modelo não é muito aceito na literatura, pois parece não reproduzir a sintomatologia da GC, devido à baixa expressão de aldolase redutase, resultando em baixa produção de galactitol nestes animais (Leslie et al., 1996).

Pacientes com GC apresentam aumento de marcadores de estresse oxidativo e reduzida capacidade antioxidante total no sangue. Os eritrócitos de pacientes apresentam baixa concentração de proteínas totais e redução nas

atividades das enzimas Na^+, K^+ -ATPase e Mg^{2+} -ATPase (Schulpis et al., 2006, 2005). Em fibroblastos de pacientes, a GAL causa perturbações na homeostase do cálcio e aumenta os níveis de estresse reticular (Slepek et al., 2007). A glicosilação anormal de proteínas e alteração na expressão de diversos genes também foram encontrados em leucócitos de pacientes com GC (Coman et al., 2010). Corroborando com esses dados, Coss e colaboradores (2014) mostrou efeitos similares em fibroblastos tratados com GAL, sugerindo que as alterações sejam similares em diferentes tipos celulares e teciduais.

1.3.3. Diagnóstico e tratamento

O diagnóstico da GC é dado a partir de programas de triagem neonatal ou pela observação clínica da sintomatologia (Fridovich-Keil and Walter, 2008). No Brasil apenas no estado de Santa Catarina e no Distrito Federal o teste da GC é disponibilizado gratuitamente, devido à políticas públicas locais, uma vez que a triagem da GC não está inclusa na triagem neonatal obrigatória do Programa Nacional de Triagem Neonatal, conhecido popularmente como “Teste do Pezinho” (Camelo Junior et al., 2011). Porém o teste da GC pode ser requerido em diversos centros médicos do país. Vale ressaltar que a sintomatologia crônica é normalmente mais severa quando os pacientes são diagnosticados tardiamente (Elsea and Lucas, 2002; Jumbo-Lucioni et al., 2012).

Os parâmetros mais utilizados no diagnóstico da GC são: níveis de galactose-1P em eritrócitos, níveis sanguíneos de GAL, atividade da GALT em eritrócitos e/ou identificação da mutação nos dois alelos que codificam a enzima

GALT. Além disso, testes moleculares têm sido aplicados para a validação da mutação (Fridovich-Keil and Walter, 2008).

O tratamento usual é a restrição de GAL da dieta. A remoção total é inviável, uma vez que diversos alimentos contêm resíduos de GAL. Nesse contexto, diferentes fórmulas de leite livre de GAL foram criadas para substituir o leite materno (Bosch, 2011; Jumbo-Lucioni et al., 2012). No entanto, a restrição dietética não é suficiente para prevenir os sintomas neurológicos que se desenvolvem ao longo da vida (Bosch, 2011; Fridovich-Keil and Walter, 2008).

1.3.4. Modelos experimentais de galactosemia clássica

O uso de camundongos geneticamente modificados é uma importante ferramenta, na obtenção de informações para a compreensão de doenças metabólicas. Porém em alguns modelos o fenótipo desenvolvido pelos animais não mimetizam plenamente a patologia humana (Elsea and Lucas, 2002). Um exemplo é o modelo experimental de GC desenvolvido por Leslie e colaboradores (1996). Nesse modelo, os animais apresentam atividade hepática da GALT quase nula, resultando em acúmulo de GAL, galactose 1-P e galactitol. Porém esses animais não apresentam complicações neurológicas e ovarianas, mesmo quando expostos a uma dieta rica em GAL (Ning et al., 2001, 2000).

Recentemente um modelo experimental nocaute da enzima GALT em *Drosophila melanogaster* foi desenvolvido (Kushner et al., 2010). Nesse modelo os animais desenvolvem alterações muito semelhantes as dos pacientes, como estresse oxidativo (Jumbo-Lucioni et al., 2013) e problemas motores (Ryan et al.,

2012). No entanto, também são observadas alterações de estresse oxidativo semelhantes ao modelo nocaute quando *Drosophila melanogaster* selvagem é exposta a GAL (Jumbo-Lucioni et al., 2013), o que faz da GAL um importante agente na investigação da fisiopatologia da GC. Um estudo mostrou que a GAL causa uma diminuição da atividade da Na^+, K^+ -ATPase e alteração de marcadores de estresse oxidativo em cérebro de ratos *in vitro* (Tsakiris et al., 2005).

Além disso, a administração de GAL tem sido usada como um modelo experimental para investigar os mecanismos envolvidos no envelhecimento, desde que foi mostrado que esse açúcar pode acelerar esse processo em ratos (Li et al., 2014) e camundongos (Parameshwaran et al., 2010). Esse modelo tem explicado vários mecanismos de toxicidade promovidos pela GAL, porém é necessário ter cautela quando extrapolar estes resultados para a GC, uma vez que os estudos não se preocuparam em medir concentrações cerebrais de GAL. É também importante mencionar que este modelo torna a barreira cérebro sangue mais permeável (Liu et al., 2015).

É importante salientar que mesmo após mais de 50 anos da descoberta da GC, os mecanismos pelos quais a GAL e/ou seus metabolitos promovem toxicidade a nível celular ainda não estão bem estabelecidos (Lai et al., 2009). Além disso, não há estudos na literatura mostrando os efeitos agudos da administração de GAL no cérebro. Desse modo é muito importante o desenvolvimento de novos modelos experimentais que auxiliem na investigação e no entendimento dos mecanismos de toxicidade da GAL na GC, para que no futuro terapias adjuvantes possam ser adotadas junto ao tratamento convencional dessa doença.

1.4. Memória

A literatura classifica a memória em dois subtipos (declarativa e não declarativa), o primeiro é conhecido como memória declarativa, que é a coleção de lembranças conscientes. A memória declarativa pode ser descrita-verbalmente, por outro lado, a memória não declarativa é composta por lembranças não conscientes, como: reflexos, hábitos e o simples condicionamento de tarefas do dia-a-dia (Izquierdo et al., 2002; Squire, 1992). Basicamente, existem três etapas para o estabelecimento de uma memória: a aquisição, (proporcionada por toda e qualquer nova experiência), a consolidação (que é o processamento da informação) e a evocação (definida pelo uso da informação adquirida) (Izquierdo and Medina, 1997).

É importante salientar que a memória pode ser armazenada por curto período (poucas horas), conhecido como memória de curta duração, ou por longos períodos (de dias até anos), conhecido como memória de longa duração. A primeira envolve potenciação sináptica de longa duração, a qual é causada por modificações em proteínas locais, como fosforilação, por exemplo. Por outro lado, a memória de longa duração envolve modificações a nível sináptico e nuclear, como expressão gênica e síntese proteica. Ambas as modificações, de curta ou longa duração, são conhecidas como plasticidade sináptica (Izquierdo et al., 2002; McGaugh, 2000). Assim, biologicamente a memória é vista como uma série de modificações a nível molecular e celular (Giovannini et al., 2015).

O hipocampo tem sido apontado como uma estrutura crucial no processamento da memória, desde a aquisição até a evocação (Izquierdo and Medina, 1997; Squire, 1992), como vem sendo mostrado em testes comportamentais que avaliam a memória não-aversiva (Antunes and Biala, 2012) e aversiva (Colettis et al., 2014) realizados em modelos animais. Prejuízos na memória também podem estar relacionados a danos no córtex cerebral (Deiana et al., 2011). Em humanos convencionalmente são aplicados testes de escrita e fala para avaliar a memória, entretendo animais não possuem tais habilidades, o que faz do uso de testes alternativos uma importante ferramenta para avaliar a memória (Antunes and Biala, 2012). No presente trabalho avaliamos o efeito da administração intracerebroventricular da GAL sobre a memória de longa duração na tarefa de reconhecimento de objetos e esQUIVA INIBITÓRIA.

1.5. Acetilcolinesterase

A acetilcolinesterase (AChE) está ancorada no exterior das membranas celulares e finaliza a transmissão colinérgica, a partir da clivagem de acetilcolina em colina e ácido acético (Pohanka, 2011). Estudos mostraram que o sistema colinérgico hipocampal desempenha um papel crucial nos processos de memória e aprendizado, participando ativamente nos mecanismos de plasticidade sináptica (Drever et al., 2011; Kukolja et al., 2009) em todas as etapas da memória, desde a formação até a evocação (Giovannini et al., 2015; Izquierdo and Medina, 1997). Nesse contexto, a AChE tem recebido atenção em diversos estudos devido a sua

função reguladora da transmissão colinérgica (Colettis et al., 2014; Mushtaq et al., 2014; Scherer et al., 2014).

Estudos têm correlacionado o funcionamento do sistema colinérgico com apoptose, proliferação celular e diferenciação neuronal (Resende and Adhikari, 2009), desde que disfunções nesse sistema possam desempenhar importantes papéis na patologia de diferentes disfunções motoras (Quik et al., 2014). Sabe-se também que a acetilcolina contribui para a produção do fator neurotrófico derivado do encéfalo (BDNF) que é responsável pela sobrevivência e proliferação celular (Resende and Adhikari, 2009). O uso de inibidores da AChE desacelera o processo neurodegenerativo e aumenta a produção de BDNF na doença de Alzheimer, a qual apresenta hipofunção colinérgica (Bendix et al., 2014).

1.6. Fator neurotrófico derivado do encéfalo

O BDNF é umas das três neurotrofinas do SNC. Além disso, o BDNF é distribuído e tem ação na periferia (He et al., 2013). A produção deste fator no SNC é regulada pela atividade neuronal (Zafra et al., 1992). O BDNF quando maduro liga-se aos receptores de TrkB na superfície neuronal, levando à diferentes respostas de sinalização celular como: regulação do desenvolvimento e plasticidade neuronal, potenciação de longa duração e atuando como um fator antiapoptótico (Chen et al., 2008; He et al., 2013; Lu, 2003).

Diversos estudos têm demonstrado que em doenças neurodegenerativas os níveis reduzidos de BDNF desempenham um importante papel na sintomatologia desenvolvida (Zuccato and Cattaneo, 2009). Em doenças que afetam o sistema motor, baixos níveis de BDNF são encontrados nas estruturas responsáveis pela coordenação do movimento como o cerebelo (He et al., 2013). No cerebelo é bem conhecido o papel do BDNF no desenvolvimento e posicionamento dos neurônios granulares e das células de Purkinje (Schwartz et al., 1997).

1.7. Coordenação motora e cerebelo

O controle da coordenação motora em condições fisiológicas envolve uma série de circuitos cerebrais e periféricos (Quik et al., 2014) e ainda pouco se conhece sobre a circuitaria necessária para a correta coordenação do movimento (Apps and Garwicz, 2005). Nesse contexto, o cerebelo parece uma estrutura central, contribuindo no sincronismo e aquisição sensorial, além de participar na predição de consequência sensorial em resposta a ação (Apps and Garwicz, 2005; Manto et al., 2012). Vale ressaltar que o cerebelo recentemente tem sido apontado estar envolvido em diversas funções cognitivas superiores (Ramnani, 2006). Lesões cerebelares podem provocar problemas de equilíbrio e postura, desordem nos movimentos dos membros e também desordens cognitivas (Manto et al., 2012).

No córtex cerebelar as células distribuem-se em três camadas: molecular, granular e Purkinje (Ramnani, 2006). Anatomicamente, o cerebelo é formado por

três regiões distintas: arquicerebelo, espinocerebelo e cerebrocerebelo. A função da primeira é a regulação do equilíbrio e movimentos dos olhos, a segunda controla o movimento do corpo e membros, enquanto a última é responsável pelo planejamento dos movimentos (Kandel et al., 2013).

Diversos testes estão disponíveis para avaliar a coordenação motora em roedores, sendo que cada teste avalia diferentes aspectos (Brooks and Dunnett, 2009). O teste da trave “*beam walking*” permite a avaliação da coordenação motora fina e equilíbrio. Diferentes condições podem provocar alterações na habilidade motora de roedores tais como: injúria cerebral, manipulação genética ou farmacológica (Brooks and Dunnett, 2009; Luong et al., 2011; Wu and Gorantla, 2014). No presente trabalho optamos utilizar o teste da trave, devido a sua sensibilidade em avaliar coordenação motora fina, a qual é prejudicada na patologia da GC.

1.8. Radicais livres e estresse oxidativo

Radical livre é qualquer átomo ou molécula com um ou mais elétrons desemparelhados. As espécies reativas mais comuns em sistemas biológicos são as derivadas do metabolismo do oxigênio e são produzidas durante o metabolismo. Vale lembrar que os radicais livres podem ter efeitos benéficos ou deletérios em sistemas biológicos como sinalização celular e danos a biomoléculas respectivamente (Halliwell, 2011, 2006; Valko et al., 2007).

Estresse oxidativo é definido por um desequilíbrio entre a produção de radicais livres e a eliminação destes pelas defesas antioxidantes, tendendo a um estado pró oxidativo, tal situação pode danificar diferentes biomoléculas a ponto de provocar perda da função celular. As biomoléculas mais susceptíveis ao ataque dos radicais livres são: proteínas, DNA e lipídeos (Halliwell, 2012; Valko et al., 2007).

As defesas antioxidantes são divididas em enzimáticas e não enzimáticas (Halliwell, 2011). A superóxido dismutase, catalase e a glutathione peroxidase são as enzimas mais estudadas e a principal frente de defesa enzimática contra os radicais livres (Halliwell, 2012). Entre as defesas não enzimáticas o tripeptídeo glutathione (GSH) se destaca devido ao seu papel de manter a homeostase redox e reparar proteínas. Tem sido mostrado que baixos níveis cerebrais de GSH estão associados com o processo de envelhecimento e doenças neurodegenerativas (Aoyama and Nakaki, 2015). Além disso, reduzidos níveis de GSH também estão associados com o processo de morte celular por apoptose (Circu and Aw, 2012; Franco and Cidlowski, 2009) e danos cerebelares que podem levar a atrofia e problemas motores (Doss et al., 2015; Franco et al., 2006; Ristoff and Larsson, 2007).

1.9. Morte celular

Necrose e apoptose são os principais processos de morte celular (Nikoletopoulou et al., 2013). Ambos podem fazer parte de processos

neurodegenerativos em fase aguda e/ou crônica (Gorman, 2008; Heimfarth et al., 2013). A necrose, também conhecida por morte celular não programada, comumente ocorre em situações de baixo nível energético e pode desencadear um processo inflamatório (Hongmei, 2012; Nikolettou et al., 2013). Enquanto isso, a apoptose é conhecida como morte celular programada e ocorre com gasto energético. Esse processo preserva a população celular vizinha por não desencadear processo inflamatório. No entanto, a apoptose é também um processo fisiológico que pode ocorrer em todos os estágios da vida de um organismo em ordem de manter as populações teciduais em homeostase. Por outro lado, a sinalização apoptótica exacerbada pode provocar injúria tecidual (Elmore, 2007; Hongmei, 2012; Nikolettou et al., 2013).

A apoptose pode ser ativada por mecanismos de sinalização celular internos (intrínseca) ou externos (extrínseca). O processo de apoptose normalmente envolve proteínas caspases, porém pode ocorrer também sem o envolvimento destas. A caspase-3 é uma proteína que ocupa uma posição central, onde convergem cascatas apoptóticas de origem intrínsecas e extrínsecas (Hongmei, 2012). Diversos fatores podem controlar o processo apoptótico, incluindo o BDNF. Nesse contexto, tem sido mostrado que baixos níveis de BDNF estão associados à morte celular (He et al., 2013). Um estudo mostrou que estresse oxidativo, disfunção mitocondrial, inflamação e apoptose estão envolvidos à fisiopatologia de diferentes EIM (Olsen et al., 2015).

2. OBJETIVOS

2.1. Objetivo geral

A fim de melhor compreender as alterações cerebrais que levam ao déficit cognitivo e a baixa coordenação motora na patologia da GC, o objetivo geral do presente estudo foi investigar a nível comportamental, celular e molecular o efeito da administração intracerebroventricular de GAL em ratos Wistar.

2.2. Objetivos específicos

Os objetivos específicos encontram-se subdivididos, os quais serão apresentados na forma de artigos científicos, como segue:

➤ **Capítulo I**

- Investigar o efeito da administração intracerebroventricular de GAL sobre a memória aversiva;
- Investigar o efeito da administração intracerebroventricular de GAL sobre a memória de reconhecimento de objetos;
- Investigar o efeito da administração intracerebroventricular de GAL sobre AChE (atividade, imunoconteúdo e expressão gênica) em hipocampo e córtex cerebral de ratos.

➤ **Capítulo II**

- Avaliar a coordenação motora de ratos, após a administração intracerebroventricular de GAL;
- Realizar contagem de células em cerebelo de ratos, após a administração intracerebroventricular de GAL;
- Investigar o efeito da administração intracerebroventricular de GAL sobre a imunofluorescência de neurônios e astrócitos em cerebelo de ratos;
- Avaliar alguns parâmetros bioquímicos tais como: níveis de GSH, dano a proteínas e DNA, marcadores de apoptose e AChE (atividade e imunoconteúdo), em cerebelo de ratos submetidos à injeção intracerebroventricular de GAL.

3. METODOLOGIA E RESULTADOS

Os procedimentos experimentais e resultados serão apresentados na forma de artigos científicos, todas as informações referentes ao desenho experimental estão contidas nos capítulos I e II.

3.1. Modelo Experimental

Ratos Wistar de 60 dias de idade foram anestesiados com ketamina/xilazina: 75 mg/kg e 10 mg/kg respectivamente. Esses animais foram à cirurgia estereotáxica com o implante cânula, dois dias após a cirurgia, uma agulha (0,9 mm) foi inserida no interior da cânula, permitindo a injeção de 5 μ L de GAL (4 mM) ou salina diretamente no terceiro ventrículo destes animais. As coordenadas relativas do ventrículo, (AP, -0.9 mm; L, -1.5 mm; DV, -2.6 mm) utilizando o Bregma como ponto de partida, foram baseadas no atlas de Paxinos and Watson (1986). Pacientes não tratados podem acumular concentrações de GAL na faixa de millimolar no cérebro (Berry, 2011). A concentração de GAL foi escolhida baseada em estudos prévios de Tsakiris e colaboradores (2002, 2005). Os animais foram decapitados 1 h, 3 h ou 24 h após a administração das substâncias.

3.2. Capítulo I

Intracerebroventricular D-galactose administration impairs memory and alters activity and expression of acetylcholinesterase in the rat

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Abstract

Tissue accumulation of galactose is a hallmark in classical galactosemia. Cognitive deficit is a symptom of this disease which is poorly understood. The aim of this study was to investigate the effects of intracerebroventricular administration of galactose on memory (inhibitory avoidance and novel object recognition tasks) of adult rats. We also investigated the effects of galactose on acetylcholinesterase (AChE) activity, immunocontent and gene expression in hippocampus and cerebral cortex. Wistar rats received a single injection of galactose (4 mM) or saline (control). For behavioral parameters, galactose was injected 1 h or 24 h previously to the testing. For biochemical assessment, animals were decapitated 1 h, 3 h or 24 h after galactose or saline injection; hippocampus and cerebral cortex were dissected. Results showed that galactose impairs the memory formation process in aversive memory (inhibitory avoidance task) and recognition memory (novel object recognition task) in rats. The activity of AChE was increased, whereas the gene expression of this enzyme was decreased in hippocampus, but not in cerebral cortex. These findings suggest that these changes in AChE may, at least in part, lead to memory impairment caused by galactose. Taken together, our results can help understand the etiopathology of classical galactosemia.

Key words: classical galactosemia; memory; acetylcholinesterase activity, gene expression.

1. Introduction

Classical galactosemia (OMIM #230400) is a genetic disorder caused by recessive mutations in the gene encoding the enzyme galactose-1-phosphate uridylyltransferase (GALT, EC 2.7.7.12) and about 200 mutations were found in humans (Calderon et al., 2007; Fridovich-Keil and Walter, 2008). These mutations decrease or null the GALT activity leading to the accumulation of galactose (GAL), galactose-1-P and galactitol (Berry, 2011; Mahmood et al., 2012; McCorvie and Timson, 2011), metabolites believed to cause cellular toxicity in brain and other tissues (Fridovich-Keil and Walter, 2008; Fridovich-Keil, 2006; Ridel et al., 2005). GAL is the first metabolite in the pathway (Fridovich-Keil and Walter, 2008) and is found in high concentration in liver and brain of untreated patients (Berry, 2011).

The first signs of the disease appear in the first weeks of life and include diarrhea, vomiting, hepatomegaly, jaundice, septicemia and kidney malfunction; untreated newborns might die within the firsts days of life (Fridovich-Keil and Walter, 2008; Holton, 1996; Waggoner et al., 1990). The main treatment is dietary restriction of GAL, nevertheless symptoms like impaired cognitive and motor function are commonly found (Fridovich-Keil and Walter, 2008; Fridovich-Keil et al., 2011; Jumbo-Lucioni et al., 2013; Kaufman et al., 1995; Potter et al., 2013). However, Berry and colleagues (1995) have shown that even patients following a GAL restrict diet might present increased GAL circulating levels due to endogenous production, a finding possibly associated to brain damage.

As the exact mechanism behind the pathology of classical galactosemia remains obscure (Jumbo-Lucioni et al., 2013), an experimental murine model of

this condition has been described where GAL levels are similar to those found in the plasma of patients with classical galactosemia. However this has been considered a poor model in reproducing the common complications found in the suckling period, like the behavioral dysfunction (Leslie et al. 1996 ; Ridel et al., 2005). Moreover, GAL was found to promote oxidative stress and a decrease of life span in the drosophila GALT *knockout* model (Jumbo-Lucioni et al., 2013).

The cholinergic system plays a key role in the modulation of learning and memory in mammals (Kukulja et al., 2009) and has also been associated with inflammation processes (Pavlov et al., 2009; Scherer et al., 2014). Acetylcholinesterase (AChE), the enzyme responsible for the termination of cholinergic transmission, is attached to the outside of the plasmatic membrane (Pohanka, 2011) and its hydrolytic activity may be altered by oxidative stress (Melo et al., 2003).

In the present study, we investigated the effects of an intracerebroventricular administration of GAL on the memory of inhibitory avoidance and novel object recognition tasks, as well as on the activity, the immunocontent and gene expression of AChE in the hippocampus and the cerebral cortex of Wistar rats. These cerebral structures were chosen because of their roles in memory modulation (Izquierdo and Medina, 1997) and their involvement in chemical models of inborn errors of metabolism using wild-type rats (Ferreira et al., 2011; Schweinberger et al., 2014; Stefanello et al., 2011; Streck et al., 2002; Vuaden et al., 2016; Wyse et al., 1995).

2. Material and methods

2.1. Animals and ethics approval

Sixty-days-old male Wistar rats were used; all animals were obtained from the Central Animal House of Biochemistry Department of Universidade Federal do Rio Grande do Sul (UFRGS). Rats were kept on a 12:12 h light-dark cycle in an air-conditioned room under constant temperature ($22\pm 1^{\circ}\text{C}$), with free access to water and 20% (w/w) protein commercial chow. All animal procedures were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH publication number 80-23 revised 1996) and the Brazilian Society for Neuroscience and Behavior recommendations for animal care. The research project was previously approved by the Ethics Committee of the University under the protocol number 27786.

2.2. Stereotaxic surgery and cannula placement

Rats were anesthetized with ketamine and xylazine (75 and 10 mg/kg i.p., respectively) and placed in a rodent stereotaxic apparatus. Under stereotaxic guidance, a 27-gauge stainless cannula (0.9 mm o.d.) with an inner needle guide was inserted unilaterally into the right ventricle (coordinates relative from bregma: AP, -0.9 mm; L, -1.5 mm; DV, -2.6 mm) (Paxinos et al., 1985). The cannula was fixed to the skull with dental cement. Two days after surgery, a 30-gauge needle was inserted into the guide cannula in order to inject 5 μL of D-galactose (4 mM) diluted in saline or vehicle (saline) into the right ventricle, over a 5 min interval.

The tip of the infusion needle protruded 1.0 mm beyond the guide cannula towards the right cerebral ventricle. Animals were randomly divided into two groups: group 1 (vehicle treated), rats that received intracerebroventricular saline and group 2 (GAL treated), rats that received intracerebroventricular GAL solution (Bavaresco et al., 2008). Since untreated patients accumulate millimolar concentration of GAL in the brain (Berry, 2011), we have chosen to adjust the GAL concentration based on previous reports of Tsakiris and colleagues (2002, 2005). For biochemical assays, animals were decapitated 1 h, 3 h or 24 h after drug administration, the brain was removed and cerebral structures (hippocampus and cerebral cortex) were dissected out.

2.3. Behavioral procedures

All behavioral procedures were performed between 10 a.m. to 3 p.m. in a controlled light and sound room, by a researcher blind to the animal's experimental condition. For the assessment of cognitive parameters (step-down inhibitory avoidance and novel object recognition) GAL was injected 1 h before the training session, in order to evaluate the process of memory acquisition. The test session was performed 24 h after the training to assess long-term memory (Bavaresco et al., 2008).

2.3. 1. Step-down inhibitory avoidance

Animals were subjected to training and test sessions in a step-down inhibitory avoidance task with an interval of 24 h between the sessions. The task was performed in an automatically operated, brightly illuminated box. The left extreme of the grid was covered by a 7.0-cm-wide, 2.5-cm-high formic platform. In the training session, animals were placed on the platform and their latencies to step down, placing their four paws on the grid (42.0 x 25.0 cm grid of parallel 0.1-cm caliber stainless steel bars spaced 1.0 cm apart), was measured. In the test session, no foot shock was delivered and step-down latency (with a ceiling of 180s) was used as a measure of memory retention as described by Wyse and colleagues (2004).

2.3.2. Novel object recognition

One day before the training session, all animals were habituated to walk freely in the empty arena for 10 min. The arena used was a black wooden box (50x50x50 cm). In the training session, two identical objects were placed equidistant from the sidewalls. In this chamber, each animal performed a trial of 10 min. After each trial, the apparatus was cleaned to alleviate olfactory cues. In the second trial, the test session, one of the objects was substituted by a different. An experimenter registered the time of object exploration, i.e., touching it with paws or exploring it by olfaction with direct contact of the snout (Plamondon et al., 2006). The object discrimination index was calculated in the test session, as follows: the difference in exploration time divided by the total time spent exploring the two objects $\{[(B-A)/(A+B)]$ where B is the new object and A is the familiar object}. This

task was performed according to Pereira and colleagues (2008) and Rojas and colleagues (2013).

2.4. Biochemical analyses

2.4.1. AChE activity assay

Hippocampus and cerebral cortex were homogenized in ten volumes (1:10; w/v) of 0.1 mM potassium phosphate buffer, pH 7.5 and centrifuged for 10 min at 1.000 g. Hydrolysis rates were measured at acetylcholine concentration of 0.8 mM in 300 μ L assay solution with 30 mM phosphate buffer, pH 7.5, and 1.0 mM 5,5'-dithiobis-(2- nitrobenzoic acid) (DTNB) at 25°C. Supernatant from homogenized tissue was added to the reaction mixture and pre incubated for 3 min. The hydrolysis was monitored by formation of the thiolate dianion of DTNB at 412 nm for 2–3 min (intervals of 30 s) (Ellman et al., 1961), with modifications (Delwing et al., 2003; Scherer et al., 2010). All samples were run in triplicate.

2.4.2. AChE Western blot

Hippocampus and cerebral cortex were homogenized in lysis solution (2 mM EDTA, 50 mM Tris-HCl, pH 6.8, plus 4% SDS) and a part of samples was taken for protein determination. The remainder was mixed (v/v) in laemmli buffer (40% glycerol, 5% mercaptoethanol, 50 mM Tris–HCl, pH 6.8) and then boiled for 3 min. In this analysis, samples were loaded (30 μ g of protein/lane) in 10%

polyacrylamide SDS-PAGE. The proteins were then transferred (Trans-blot SD semidry transfer cell; Bio-Rad) to nitrocellulose membranes, for 1 h at 15 V in transfer buffer (48 mM Trizma, 39 mM glycine, 20% methanol and 0.25% SDS). The membrane was washed during 10 min in Tris buffer saline (TBS; 500 mM NaCl, 20 mM Trizma, pH 7.5). The block of the membrane was performed with TBS plus 5% bovine serum albumin (BSA), for 2 h. Afterwards the membrane was washed twice during 5 min in (T-TBS; TBS plus 0.05% Tween- 20). Subsequently it was incubated overnight at 4°C in a blocking solution plus AChE antibody polyclonal anti-rabbit IgG (1:1000; Santa Cruz Biotechnology). Then the membrane was washed twice for 5 min with T-TBS and incubated for 2 h in antibody solution containing peroxidase-conjugated anti-rabbit IgG (1:2000; Santa Cruz Biotechnology). In the next step, the membrane was washed twice again for 5 min with T-TBS and twice for 5 min with TBS. The membrane was developed using chemiluminescent ECL kit (Immobilon Western Chemiluminescent HRP Substrate, Millipore) and detected by ImageQuant LAS 4000 (GE Healthcare Life Sciences). This technique was performed as described by Scherer and colleagues (2014). The Western blot analyses were performed twice.

2.4.3. Gene expression of AChE by quantitative real time RT-PCR

The analysis of AChE expression was performed as described by Scherer and colleagues (2014) by quantitative real time reverse transcription polymerase chain reaction (RT-PCR) using SYBR Green I (Molecular Probes) as the fluorescent detector. Gene sequences of AChE and the housekeeping gene

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were acquired from databases (www.ncbi.nlm.nih.gov and www.ensembl.org) and used for primers' design with free software available from www.idtdna.com Table 1. Samples from hippocampus or cortex were dissected, immediately frozen in liquid nitrogen and stored at -80°C for posterior use. mRNA was extracted using TRIzol® reagent (Life Technologies) in accordance with the manufacturer instructions. cDNA synthesis was carried out with M-MLV Reverse transcriptase (SIGMA) from 2 µg of total RNA. 2 µL of diluted cDNA (1:20) was used as template for PCR reactions with Platinum® Taq Polymerase (Life Technologies) in a final volume of 20 µL. The thermal cycling profile for all genes was an initial denaturation step at 94°C for 10 min followed by 40 cycles of 15 s at 94°C, 15 s at 60°C, 15 s at 72°C for data acquisition. Reaction specificity and absence of primer-dimer formation was evaluated using melting curve analysis at the end of each run. Finally, we confirmed the presence of a single amplicon of the specified size by agarose gel electrophoresis. All reactions were carried out in a StepOnePlus® real-time PCR system (Applied Biosystems). Samples relative ratios were calculated by the comparative CT method ($\Delta\Delta$ CT method) (Livak and Schmittgen, 2001), normalized by the housekeeping gene (GAPDH) and calibrated by the average of the Δ CT of the group. Reaction efficiencies of both genes (AChE and GAPDH) were similar.

2.4.4. Protein determination

The amount of protein in samples was estimated by the method described by Bradford (1976). For this procedure, a standard curve of purified albumin was performed.

2.5. Statistical analysis

Differences between test and training latency on inhibitory avoidance task were assessed by individual (two tailed) Mann–Whitney U tests. Descriptive statistics data were expressed as median (interval interquartile). Data from novel object recognition and biochemical tests were evaluated using Student's *t* test and results were presented as mean \pm S.E.M. or mean \pm S.D respectively. For all tests $p < 0.05$ was assumed as statistically significant. All analyses were performed with the software Statistical Package for the Social Sciences (SPSS; v.20).

3. Results

3.1. GAL impairs long-term memory formation

Firstly, we evaluated the effect of GAL on long-term aversive memory. In the training session (acquisition) 1 h after GAL infusion no differences in latency were observed between control and treated animals ($U = 40.50$; $p > 0.05$). In the test

session (retrieval) 24 h after the training the group which received GAL infusion exhibited a lower latency in comparison to the control group ($U = 13.00$; $p < 0.01$) (Fig. 1).

The novel object recognition test was applied to assess the effect of GAL on recognition memory. GAL was injected 1 h before training session, in which no differences were observed in the time exploring the object or in the recognition index ($p > 0.05$). The time exploring the novel object increased within the control group [$t(24) = 2.983$; $p < 0.01$], but it remained unchanged in treated animals [$t(30) = 0.9892$; $p > 0.05$] (Fig. 2A). The group treated with GAL also exhibited a reduced discrimination index [$t(27) = 2.243$; $p < 0.05$] (Fig. 2B).

3.2. GAL alters AChE activity and gene expression

GAL increased the activity of AChE at 1 h [$t(10) = 2.592$; $p < 0.05$] and 3 h [$t(10) = 4.201$; $p < 0.01$], but 24 h after administration this enzyme activity returned to normal levels in the hippocampus [$t(12) = 0.3932$; $p > 0.05$] (Fig 3A). In cerebral cortex, GAL did not alter AChE activity in none of times tested ($p > 0.05$) (Fig 3B).

GAL did not alter the immunoccontent of AChE in hippocampus or cerebral cortex (*data not shown*), but this compound decreased AChE mRNA expression in the hippocampus at 3 h after injection [$t(10) = 4.005$; $p < 0.01$] (Fig. 4A), but not its immunoccontent [$t(11) = 0.5681$; $p > 0.05$] (Fig. 4B). In cerebral cortex, the expression of this enzyme was not changed ($p > 0.05$).

4. Discussion

In the present study we showed the effects of intracerebroventricular GAL injection on aversive and novel object recognition memories. We observed that the injection of GAL 1 h before training session (acquisition phase) impairs memory in both tested tasks, confirming that the experimental model used is capable of reproducing memory dysfunction, which is commonly found in galactosemic patients (Fridovich-Keil and Walter, 2008; Ridel et al., 2005).

The cholinergic system can modulate cognition, emotion and brain electrical activity (Graef et al., 2011). Cholinergic transmission is essential because it takes part in the process of memory formation and consolidation even after long time periods (Giovannini et al., 2015; Izquierdo and Medina, 1997). AChE participates in the end of cholinergic transmission breaking down acetylcholine in choline and acetic acid (Pohanka, 2011). Based on this, we also tested the effect of GAL on AChE. Our results showed an increase in AChE activity in hippocampus at 1 h and 3 h after GAL injection. The immunocontent remained unaltered 3 h after the injection, whereas the gene expression was decreased, suggesting a homeostatic recovery of AChE activity observed 24 h after GAL injection. However, alternative splicing may occur under stress conditions leading to the production of readthrough AChE, which is barely expressed in physiological conditions (Meshorer and Soreq, 2006; Meshorer et al., 2002). In this study, we have investigated the synaptic AChE mRNA. Future investigations are necessary to evaluate the readthrough AChE expression in models of GAL toxicity. These changes in AChE suggest that GAL may impair the cholinergic system in hippocampus, which could lead to deficit

in memory formation (Mushtaq et al., 2014). In cerebral cortex, we did not observe changes on AChE activity. More studies are necessary to understand the effect of GAL on AChE in different cerebral structures.

It has been shown that AChE activity is increased in some diseases that affect the brain, including Alzheimer disease (Mushtaq et al., 2014) which seems to be associated with memory deficits. Similar results were found applying the aging model, which uses chronic GAL exposure (Lu et al., 2010). Other *in vitro* studies demonstrate that GAL did not alter AChE activity in rat brain homogenates (Marinou et al., 2005; Tsakiris and Schulpis, 2000), but these authors suggest these results may be due to cellular context loss in homogenates.

It is crucial to understand the mechanisms behind the neuropathology of classical galactosemia to develop more efficient therapeutic strategies. The current treatment is based on GAL restrict diet, which does not prevent the neurological symptoms of classical galactosemia (Calcar et al., 2014; Ridel et al., 2005). In agreement with our results, changes in the cholinergic system have been described in an aging model that utilizes a load of GAL (Lu et al., 2010), but in this study the concentrations of GAL are unknown in tissues. Considering that little is known about the effects of GAL in the central nervous system, our study seems to be relevant since GAL levels were similar to those found in brain of patients affected by classical galactosemia.

5. Conclusions

In summary, the present study demonstrated that a single intracerebroventricular GAL injection impaired aversive and recognition memory in adult rats. Activity and gene expression of AChE were altered, which may be associated with the memory impairment caused by GAL. We believe that these findings may help understand the physiopathology behind the neurological symptoms of classical galactosemia and open a new approach to study the disease.

Conflict of interest

The authors declare to have no conflict of interest.

Acknowledgments

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References

- Bavaresco, C.S., Ben, J., Chiarani, F., Netto, C.A., Wyse, A.T.S., 2008. Intrastratial injection of hypoxanthine impairs memory formation of step-down inhibitory avoidance task in rats. *Pharmacol. Biochem. Behav.* 90, 594–597. doi:10.1016/j.pbb.2008.05.001
- Berry, G.T., 2011. Is prenatal myo-inositol deficiency a mechanism of CNS injury in galactosemia? *J. Inherit. Metab. Dis.* 34, 345–355. doi:10.1007/s10545-010-9260-x
- Berry, G.T., Nissim, I., Lin, Z., Mazur, A.T., Gibson, J.B., Segal, S., 1995. Endogenous synthesis of galactose in normal men and patients with hereditary galactosaemia. *Lancet* 346, 1073–1074.

- Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248–254. doi:10.1016/0003-2697(76)90527-3
- Calderon, F.R.O., Phansalkar, A.R., Crockett, D.K., Miller, M., Mao, R., 2007. Mutation database for the galactose-1-phosphate uridylyltransferase (GALT) gene. *Hum. Mutat.* 28, 939–943. doi:10.1002/humu.20544
- Delwing, D., Chiarani, F., Delwing, D., Bavaresco, C.S., Wannmacher, C.M.D., Wajner, M., Wyse, A.T.S., 2003. Proline reduces acetylcholinesterase activity in cerebral cortex of rats. *Metab. Brain Dis.* 18, 79–86.
- Ellman, G.L., Courtney, K.D., Andres, V., Featherstone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.* 7, 88–95. doi:10.1016/0006-2952(61)90145-9
- Ferreira, A.G.K., Scherer, E.B., da Cunha, M.J., Machado, F.R., da Cunha, A.A., Graeff, J.S., Netto, C.A., Wyse, A.T.S., 2011. Physical Exercise Reverses Cognitive Impairment in Rats Subjected to Experimental Hyperprolinemia. *Neurochem. Res.* 36, 2306–2315. doi:10.1007/s11064-011-0555-6
- Fridovich-Keil, J.L., 2006. Galactosemia: The good, the bad, and the unknown. *J. Cell. Physiol.* 209, 701–705. doi:10.1002/jcp.20820
- Fridovich-Keil, J.L., Gubbels, C.S., Spencer, J.B., Sanders, R.D., Land, J.A., Rubio-Gozalbo, E., 2011. Ovarian function in girls and women with GALT-deficiency galactosemia. *J. Inherit. Metab. Dis.* 34, 357–366. doi:10.1007/s10545-010-9221-4
- Fridovich-Keil, J.L., Walter, J., 2008. Galactosemia, in: Valle, D., Beaudet, A., Vogelstein, B., Kinzler, K., Antonarakis, S., Ballabio, A. (Eds.), *The Online Metabolic & Molecular Bases of Inherited Disease*. McGrawHill, pp. 1–74.
- Giovannini, M.G., Lana, D., Pepeu, G., 2015. The integrated role of ACh, ERK and mTOR in the mechanisms of hippocampal inhibitory avoidance memory. *Neurobiol. Learn. Mem.* 119, 18–33. doi:10.1016/j.nlm.2014.12.014
- Graef, S., Schönknecht, P., Sabri, O., Hegerl, U., 2011. Cholinergic receptor subtypes and their role in cognition, emotion, and vigilance control: an overview of preclinical and clinical findings. *Psychopharmacology* 215, 205–229. doi:10.1007/s00213-010-2153-8
- Holton, J.B., 1996. Galactosaemia: pathogenesis and treatment. *J. Inherit. Metab. Dis.* 19, 3–7. doi:10.1007/BF01799341
- Izquierdo, I., Medina, J.H., 1997. Memory Formation: The Sequence of Biochemical Events in the Hippocampus and Its Connection to Activity in Other Brain Structures. *Neurobiol. Learn. Mem.* 68, 285–316. doi:10.1006/nlme.1997.3799
- Jumbo-Lucioni, P.P., Hopson, M.L., Hang, D., Liang, Y., Jones, D.P., Fridovich-Keil, J.L., 2013. Oxidative stress contributes to outcome severity in a *Drosophila melanogaster* model of classic galactosemia. *Dis. Model. Mech.* 6,

84–94. doi:10.1242/dmm.010207

- Kaufman, F.R., McBride-Chang, C., Manis, F.R., Wolff, J.A., Nelson, M.D., 1995. Cognitive functioning, neurologic status and brain imaging in classical galactosemia. *Eur. J. Pediatr.* 154, S2–5.
- Kukulja, J., Thiel, C.M., Fink, G.R., 2009. Cholinergic stimulation enhances neural activity associated with encoding but reduces neural activity associated with retrieval in humans. *J. Neurosci.* 29, 8119–8128. doi:10.1523/JNEUROSCI.0203-09.2009
- Leslie, N.D., Yager, K.L., McNamara, P.D., Segal, S., 1996. A mouse model of galactose-1-phosphate uridyl transferase deficiency. *Biochem. Mol. Med.* 59, 7–12. doi:10.1006/bmme.1996.0057
- Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25, 402–408. doi:10.1006/meth.2001.1262
- Lu, J., Wu, D.M., Hu, B., Cheng, W., Zheng, Y.L., Zhang, Z.F., Ye, Q., Fan, S.H., Shan, Q., Wang, Y.J., 2010. Chronic administration of troxerutin protects mouse brain against D-galactose-induced impairment of cholinergic system. *Neurobiol. Learn. Mem.* 93, 157–164. doi:10.1016/j.nlm.2009.09.006
- Mahmood, U., Imran, M., Naik, S.I., Cheema, H.A., Saeed, A., Arshad, M., Mahmood, S., 2012. Detection of common mutations in the GALT gene through ARMS. *Gene* 509, 291–294. doi:10.1016/j.gene.2012.08.010
- Marinou, K., Tsakiris, S., Tsopanakis, C., Schulpis, K.H., Behrakis, P., 2005. Suckling rat brain regional distribution of acetylcholinesterase activity in galactosaemia in vitro. *Metab. Brain Dis.* 20, 227–236. doi:10.1007/s11011-005-7210-3
- McCorvie, T.J., Timson, D.J., 2011. Structural and molecular biology of type I galactosemia: disease-associated mutations. *IUBMB Life* 63, 949–954. doi:10.1002/iub.510
- Melo, J.B., Agostinho, P., Oliveira, C.R., 2003. Involvement of oxidative stress in the enhancement of acetylcholinesterase activity induced by amyloid beta-peptide. *Neurosci. Res.* 45, 117–127. doi:10.1016/S0168-0102(02)00201-8
- Meshorer, E., Erb, C., Gazit, R., Pavlovsky, L., Kaufer, D., Friedman, A., Glick, D., Ben-Arie, N., Soreq, H., 2002. Alternative splicing and neuritic mRNA translocation under long-term neuronal hypersensitivity. *Science* 295, 508–512. doi:10.1126/science.1066752
- Meshorer, E., Soreq, H., 2006. Virtues and woes of AChE alternative splicing in stress-related neuropathologies. *Trends Neurosci.* 29, 216–224. doi:10.1016/j.tins.2006.02.005
- Mushtaq, G., Greig, N.H., Khan, J.A., Kamal, M.A., 2014. Status of acetylcholinesterase and butyrylcholinesterase in Alzheimer's disease and type 2 diabetes mellitus. *CNS Neurol. Disord. Drug Targets* 13, 1432–14329.

- Pavlov, V.A., Parrish, W.R., Rosas-Ballina, M., Ochani, M., Puerta, M., Ochani, K., Chavan, S., Al-Abed, Y., Tracey, K.J., 2009. Brain acetylcholinesterase activity controls systemic cytokine levels through the cholinergic anti-inflammatory pathway. *Brain. Behav. Immun.* 23, 41–45. doi:10.1016/j.bbi.2008.06.011
- Paxinos, G., Watson, C., Pennisi, M., Topple, A., 1985. Bregma, lambda and the interaural midpoint in stereotaxic surgery with rats of different sex, strain and weight. *J. Neurosci. Methods* 13, 139–143.
- Pereira, L.O., Strapasson, A.C.P., Nabinger, P.M., Achaval, M., Netto, C.A., 2008. Early enriched housing results in partial recovery of memory deficits in female, but not in male, rats after neonatal hypoxia-ischemia. *Brain Res.* 1218, 257–266. doi:10.1016/j.brainres.2008.04.010
- Plamondon, H., Morin, A., Charron, C., 2006. Chronic 17beta-estradiol pretreatment and ischemia-induced hippocampal degeneration and memory impairments: a 6-month survival study. *Horm. Behav.* 50, 361–369. doi:10.1016/j.yhbeh.2006.04.006
- Pohanka, M., 2011. Cholinesterases, a target of pharmacology and toxicology. *Biomed. Pap.* 155, 219–230. doi:10.5507/bp.2011.036
- Potter, N.L., Nievergelt, Y., Shriberg, L.D., 2013. Motor and Speech Disorders in Classic Galactosemia. *JIMD Rep.* 11, 31–41. doi:10.1007/8904_2013_219
- Ridel, K.R., Leslie, N.D., Gilbert, D.L., 2005. An updated review of the long-term neurological effects of galactosemia. *Pediatr. Neurol.* 33, 153–161. doi:10.1016/j.pediatrneurol.2005.02.015
- Rojas, J.J., Deniz, B.F., Miguel, P.M., Diaz, R., Hermel, E.E.S., Achaval, M., Netto, C.A., Pereira, L.O., 2013. Effects of daily environmental enrichment on behavior and dendritic spine density in hippocampus following neonatal hypoxia–ischemia in the rat. *Exp. Neurol.* 241, 25–33. doi:10.1016/j.expneurol.2012.11.026
- Scherer, E.B.S., da Cunha, M.J., Matté, C., Schmitz, F., Netto, C.A., Wyse, A.T.S., 2010. Methylphenidate affects memory, brain-derived neurotrophic factor immunocontent and brain acetylcholinesterase activity in the rat. *Neurobiol. Learn. Mem.* 94, 247–253. doi:10.1016/j.nlm.2010.06.002
- Scherer, E.B.S., Loureiro, S.O., Vuaden, F.C., da Cunha, A.A., Schmitz, F., Kolling, J., Savio, L.E.B., Bogo, M.R., Bonan, C.D., Netto, C.A., Wyse, A.T.S., 2014. Mild Hyperhomocysteinemia Increases Brain Acetylcholinesterase and Proinflammatory Cytokine Levels in Different Tissues. *Mol. Neurobiol.* 50, 589–596. doi:10.1007/s12035-014-8660-6
- Schweinberger, B.M., Schwieder, L., Scherer, E., Sitta, A., Vargas, C.R., Wyse, A.T.S., 2014. Development of an animal model for gestational hypermethioninemia in rat and its effect on brain Na⁺,K⁺-ATPase/Mg²⁺-ATPase activity and oxidative status of the offspring. *Metab. Brain Dis.* 29, 153–160. doi:10.1007/s11011-013-9451-x

- Stefanello, F.M., Ferreira, A.G.K., Pereira, T.C.B., da Cunha, M.J., Bonan, C.D., Bogo, M.R., Wyse, A.T.S., 2011. Acute and chronic hypermethioninemia alter Na⁺,K⁺-ATPase activity in rat hippocampus: prevention by antioxidants. *Int. J. Dev. Neurosci.* 29, 483–488. doi:10.1016/j.ijdevneu.2011.02.001
- Streck, E.L., Matte, C., Vieira, P.S., Rombaldi, F., Wannmacher, C.M.D., Wajner, M., Wyse, A.T.S., 2002. Reduction of Na⁽⁺⁾,K⁽⁺⁾-ATPase activity in hippocampus of rats subjected to chemically induced hyperhomocysteinemia. *Neurochem. Res.* 27, 1593–1598.
- Tsakiris, S., Carageorgiou, H., Schulpis, K.H., 2005. The protective effect of L-cysteine and glutathione on the adult and aged rat brain (Na⁺,K⁺)-ATPase and Mg²⁺-ATPase activities in galactosemia in vitro. *Metab. Brain Dis.* 20, 87–95. doi:10.1007/s11011-005-2480-3
- Tsakiris, S., Marinou, K., Schulpis, K.H., 2002. The in vitro Effects of Galactose and its Derivatives on Rat Brain Mg²⁺-ATPase Activity. *Pharmacol. Toxicol.* 91, 254–257. doi:10.1034/j.1600-0773.2002.910506.x
- Tsakiris, S., Schulpis, K.H., 2000. The effect of galactose metabolic disorders on rat brain acetylcholinesterase activity. *Zeitschrift Fur Naturforsch C* 55, 852–855.
- Van Calcar, S.C., Bernstein, L.E., Rohr, F.J., Scaman, C.H., Yannicelli, S., Berry, G.T., 2014. A re-evaluation of life-long severe galactose restriction for the nutrition management of classic galactosemia. *Mol. Genet. Metab.* 112, 191–197. doi:10.1016/j.ymgme.2014.04.004
- Vuaden, F.C., Savio, L.E.B., Rico, E.P., Mussulini, B.H.M., Rosemberg, D.B., de Oliveira, D.L., Bogo, M.R., Bonan, C.D., Wyse, A.T.S., 2016. Methionine Exposure Alters Glutamate Uptake and Adenine Nucleotide Hydrolysis in the Zebrafish Brain. *Mol. Neurobiol.* 53, 200–209. doi:10.1007/s12035-014-8983-3
- Waggoner, D., Buist, N., Donnell, G., 1990. Long-term prognosis in galactosemia: results of a survey of 350 cases. *J Inherit Metab Dis* 13, 802–818.
- Wyse, A.T., Sarkis, J.J., Cunha-Filho, J.S., Teixeira, M. V, Schetinger, M.R., Wajner, M., Wannmacher, C.M., 1995. ATP diphosphohydrolase activity in synaptosomes from cerebral cortex of rats subjected to chemically induced phenylketonuria. *Braz. J. Med. Biol. Res.* 28, 643–649.
- Wyse, A.T.S., Bavaresco, C.S., Reis, E.A., Zugno, A.I., Tagliari, B., Calcagnotto, T., Netto, C.A., 2004. Training in inhibitory avoidance causes a reduction of Na⁺,K⁺-ATPase activity in rat hippocampus. *Physiol. Behav.* 80, 475–479. doi:10.1016/j.physbeh.2003.10.002

Legend to Figures

Fig. 1 Effect of intracerebroventricular administration of galactose, injected 1 h before training session, on step-down inhibitory avoidance. Data are median (interquartile range) for 10-11 animals in each group. ^{##}p< 0.01 Different from the control group (Mann–Whitney).

Fig. 2 Effect of intracerebroventricular administration of galactose, injected 1 h before training session, on novel object recognition. Time spent in each object (A) and discrimination index (B) in the test session. Note A is the old object and B is the novel object, and the discrimination index was calculated by the formula [(B-A)/(A+B)]. Data are expressed as mean \pm S.E.M. for 13-16 animals in each group. * p< 0.05; **p< 0.01 Different from the control group (Student's *t* test).

Fig. 3 Effect of intracerebroventricular injection of galactose on activity of acetylcholinesterase in hippocampus (A) and cerebral cortex (B) of rats at different times after injection. Data are expressed as mean \pm S.D. for 6-7 animals in each group. * p< 0.05; **p< 0.01 Different from the control group (Student's *t* test).

Fig. 4 Effect of intracerebroventricular injection of galactose on gene expression (A) and immunocontent (B) of acetylcholinesterase in hippocampus at 3 h after injection. Data are expressed as mean \pm S.D. for 6-7 animals in each group. **p< 0.01 Different from the control group (Student's *t* test).

Figures and Table

Tab.1

Table 1 - RT-PCR primers sequence of AChE and GAPDH

Gene	N° GenBank (mRNA)	N° Ensembl (gene)		Primer sequence	Amplicon size
AChE	NM_172009	ENSRNOG00000050841	Foward	CTGGGTTTGAGGGTACCGAG	114 bp
			Reverse	TGAGGACAGGTGTGGGAGAA	
GAPDH	NM_017008	ENSRNOG00000018630	Foward	GGTGATGCTGGTGCTGAGTA	272 bp
			Reverse	ACTGTGGTCATGAGCCCTTC	

Fig. 1

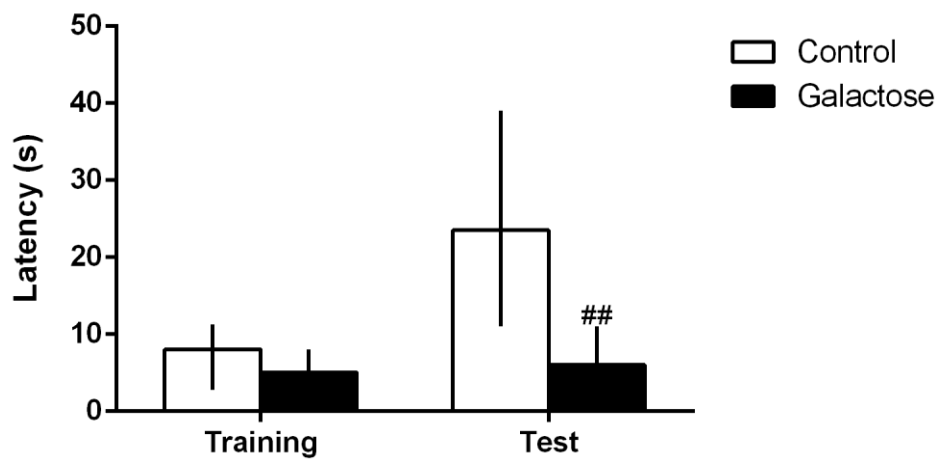


Fig. 2

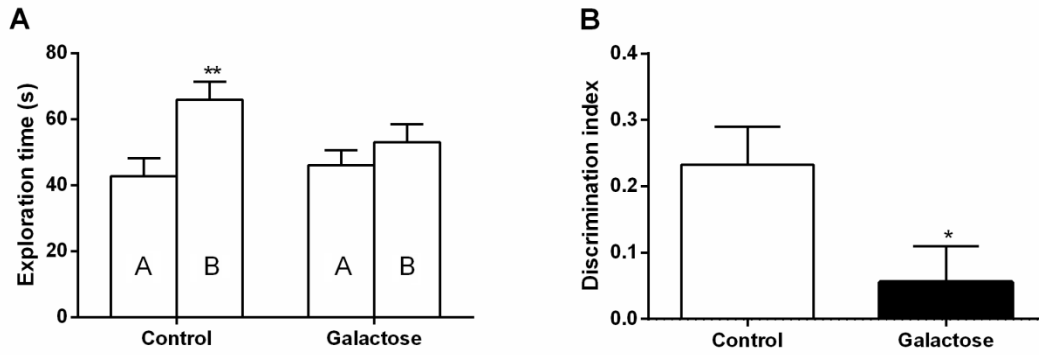


Fig. 3

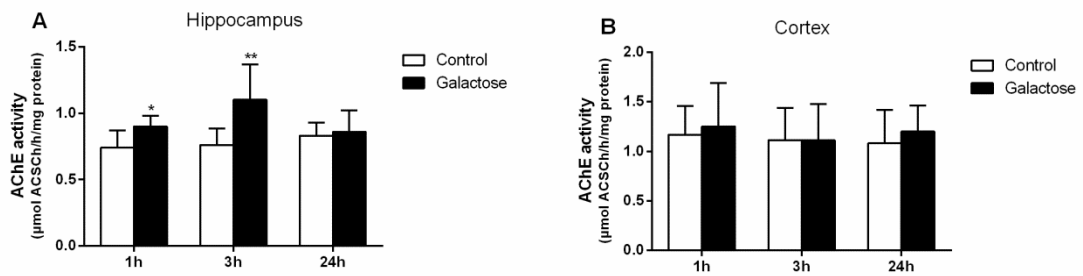
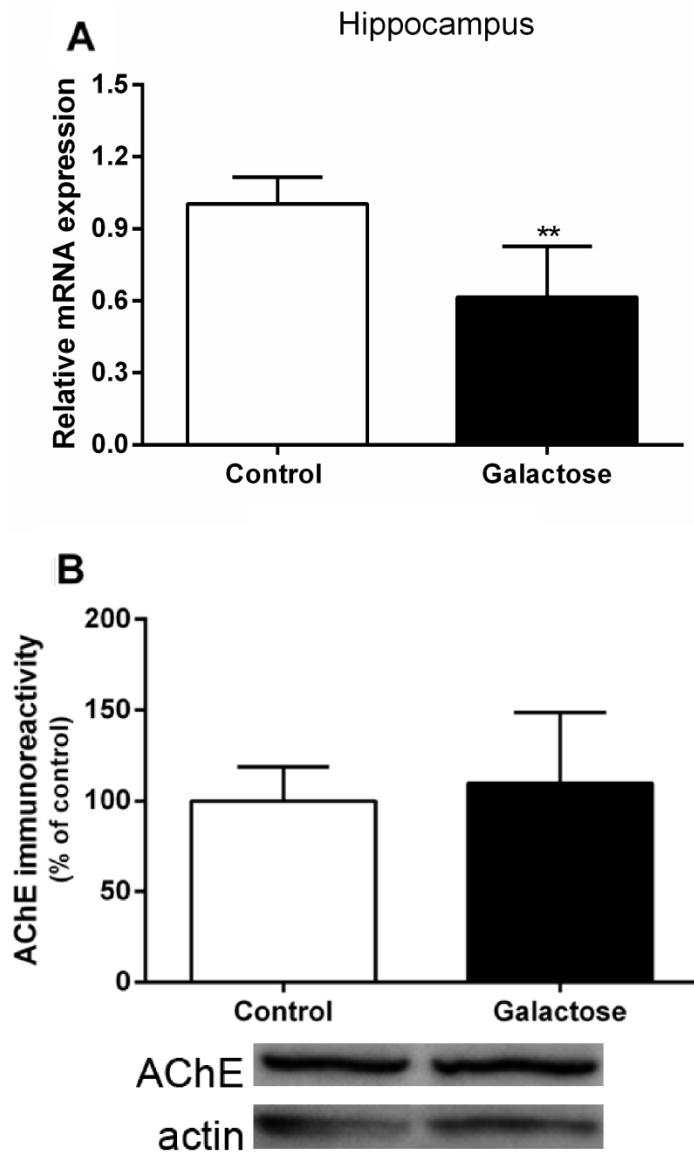


Fig. 4



3.3. Capítulo II

D-galactose causes motor coordination impairment, and histological and biochemical changes in the cerebellum of rats

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Periódico: Molecular Neurobiology

Status: A ser submetido

D-galactose causes motor coordination impairment, and histological and biochemical changes in the cerebellum of rats

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Abstract

Classical galactosemia is an inborn error of carbohydrate metabolism in which patients accumulate high concentration of galactose in the brain. The most common treatment is a galactose restricted diet. However, even treated patients develop several complications. One of the most common symptoms is motor coordination impairment, including affected gait, balance and speech as well as tremor and ataxia. In the present study, we investigated the effects of intracerebroventricular galactose administration on motor coordination, as well as on histological and biochemical parameters in cerebellum of adult rats. Wistar rats received 5 μ L of galactose (4 mM) or saline by intracerebroventricular injection. The animals performed the beam walking test at 1 h and 24 h after galactose administration. Histological and biochemical parameters were performed 24 h after the injections. The results showed motor coordination impairment at 24 h after galactose injection. Galactose also decreased the number of cells in the molecular and granular layers of the cerebellum. The immunohistochemistry results suggest that the cell types lost by galactose are neurons and astrocytes in the spinocerebellum and neurons in the cerebrocerebellum. Galactose increased active caspase-3 immunocontent and acetylcholinesterase activity, decreased glutathione levels, BDNF and acetylcholinesterase immunocontent, as well as caused protein and DNA damage. Our results suggest that galactose induces histological and biochemical changes in cerebellum, which can be associated with motor coordination impairment.

Key words: galactose; motor coordination; BDNF; caspase-3; acetylcholinesterase; apoptosis; cell damage

Introduction

Classical galactosemia is caused by different recessive mutations in the gene encoding of galactose 1-P uridil transferase [1, 2], which leads to tissue accumulation of metabolites, mainly galactose (GAL) [2, 3]. The main treatment is a GAL restrict diet [4]. However, often treated patients, develop disturbances of coordination throughout their life [5–8]. Moreover, the etiology of the disease is still poorly understood [9, 10]. Patients with classical galactosemia present alteration in markers of oxidative stress and in activities of Na⁺,K⁺-ATPase and Mg²⁺-ATPase in blood [11, 12]. The treatment of patients fibroblasts with GAL alters calcium homeostasis and causes reticular stress [13]. In this context, classical galactosemia seems to be a multi-system disorder with a wide range of altered gene expression [14].

The cerebellum plays a key role on coordination and fine movements as well as on motor learning. Cerebellar damage may lead to impairment in fine movement, balance, posture and motor learning [15, 16]. The role of the cerebellum has been shown to be beyond of the motor coordination, extending to cognitive function [17, 18]. In this perspective, cerebellar damage is supposed to trigger the coordination and speech complications of classical galactosemia [8], since coordination dysfunction affects around 70% of the galactosemic

population [7, 8]. Cerebellar atrophy is observed in patients with classical galactosemia [19, 20], also, a case of autopsy study reported several brain alterations, including cerebellar Purkinje cell loss, spared granular layer and gliosis [21].

Acute and/or chronic neurodegeneration involves cell death, primarily neuronal loss; the loss of cells is provoked by apoptosis and/or necrosis [22, 23]. The type of cell death selected depends on the stimulus and the cellular context and is a result of self-propagating signals [24]. Two distinct pathways initiate apoptosis: caspase-dependent or caspase-independent pathway [25]. In the active form, several caspases proteins lead to cell apoptosis. In this context, caspase-3 plays a central role in the intrinsic and extrinsic apoptosis caspase dependent routes [25–27] and is responsible for most of the observed changes during apoptosis, such as the enhancement of mitochondrial membrane permeability [28].

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophic family of growth factors, BDNF is widespread in the central nervous system and in the periphery [29]. The mature BDNF binds mainly to TrkB receptors, and plays an important role in cell survival processes [29, 30]. In addition, BDNF can directly protect injured neurons from apoptosis by inhibiting the activation of caspases [31]. In line with that, reduced levels of BDNF are found in neurodegenerative diseases that affect motor structures [29].

The BDNF synthesis is stimulated by neuronal activity [32]. It has been shown that in the hippocampus BDNF production is directly influenced by acetylcholine levels [33]. In this context, several studies have shown that

acetylcholinesterase (AChE) inhibition increases BDNF levels by prolonging acetylcholine action on its receptors [34, 35]. The crosstalk between the cholinergic system and BDNF production suggests an important role of the cholinergic system in cell survival [36].

Glutathione (GSH) is an endogenously synthesized antioxidant, well known for repairing thiol groups of oxidized proteins and by acting as free radical scavenger [37, 38]. GSH depletion is involved in the apoptotic processes in both intrinsic and/or extrinsic pathways [39, 40]. Also, the depletion of GSH content can cause damage to biomolecules such as proteins and DNA, which contributes to the spread of apoptotic pathways [41].

Although, it is known that cerebellar atrophy is one of the main findings in classical galactosemia, and that this may be related to motor disorders found in patients with this disease. In the present study we aimed to investigate the effects of intracerebroventricular GAL injection on motor coordination in Wistar rats, and its relationship with neurons and astrocyte loss. Biochemical parameters related with cell death, such as active caspase-3 immunocontent, BDNF levels, AChE activity/immunocontent, GSH levels, sulfhydryl levels and DNA damage, were also evaluated in cerebellum of rats.

Material and methods

Animals and ethic statements

Sixty-day-old male Wistar rats were obtained from Central Animal House of the Department of Biochemistry of the Universidade Federal do Rio Grande do Sul

(UFRGS), Porto Alegre, Brazil. The animals were randomly divided into two groups: (1) Control (saline-injected) and (2) Galactose (GAL-injected). The rats were kept on a 12 h light/12 h dark cycle at a constant temperature ($22\pm 1^{\circ}\text{C}$), with free access to water and commercial protein chow. Animal care followed the Guide for Care and Use of Laboratory Animals (NIH publication number 80-23 revised 1996) and the recommendations for animal care of the Brazilian Society for Neuroscience and Behavior. The project was approved by the local ethics committee (No. 27786).

Stereotaxic surgery and cannula placement

The animals were anesthetized with ketamine and xylazine (75 and 15 mg/kg i.p., respectively). Based on stereotaxic measures, a 27 gauge 0.9 mm diameter cannula was positioned and fixed in the rat ventricle as detailed described by Delwing and colleagues [42]. The used coordinates (AP: -0.9 mm; L: -1.5 mm; DV: -2.6 mm) were based on the atlas of Paxinos and Watson [43].

Galactose administration and tissue preparation

Each animal received a single intracerebroventricular injection ($5\ \mu\text{L}$; $1\ \mu\text{L}/\text{min}$) of GAL (4 mM) or saline 48 h after the surgery. The solutions were delivered using a 30 gauge needle with 1.0 mm protuberant tip connected by polystyrene tube to a Hamilton microsyringe. The dosage of GAL was chosen on previous reports of Tsakiris and colleagues [44, 45]. Our study is an attempt to mimic conditions of

untreated galactosemic patients, which used to accumulate millimolar concentration of GAL in the central nervous system [46].

The rats were killed 24 h after the injections. The cerebellum was dissected out on an ice-cooled dish, subsequently frozen at -80°C until the biochemical parameters determination.

Beam walking task

The behavioral procedure was performed between 10 a.m. to 1 p.m. The same investigator performed all experimental sessions, in a controlled light and sound room. To access locomotor deficits, we modified the protocol described by Lotan and colleagues [47]. One day prior GAL injection, the rats were trained (three trials) to traverse a narrow wooden beam (width; 2.5 cm, length; 100 cm). The beam was elevated 50 cm above the floor by two acrylic boxes. The animals were placed on one side, with a safe place (a black box) on the other side, encouraging the animals to walk on the beam. In the test session, the number of hindpaw slips were counted (three trials) at 1 h and 24 h after GAL infusion.

Histological assessment

After behavioral analysis, the animals were deeply anesthetized with sodium thiopental (50 mg/kg; i.p.) and transcardially perfused through the left ventricle using a peristaltic pump (Control Company, São Paulo, Brazil) with saline solution followed by 4% paraformaldehyde (PFA; Sigma-Aldrich) in 0.1 M phosphate buffer saline (PBS) pH 7.4 at room temperature. The brains were post-fixed in PFA

overnight and kept in 30% sucrose in PBS for 3 days and then frozen. Cerebellar coronal section (30 μm) were mounted on gelatinized glass slides and stained with hematoxylin and eosin. The slices containing the spinocerebellum and cerebrocerebellum were prepared based on the atlas of Paxinos and Watson [43]. The slides were visualized with a microscope (Nikon TE300; Nikon, Osaka, Japan) coupled to a CCD camera (Quantix 512-Roper Scientific Inc., Princeton Instruments, Princeton, NJ). Images were acquired in BioIP software (Anderson Eng, Delaware, USA). Ten fields of 14400 μm^2 each in the granular and molecular layers per animal were delineated in the software ImageJ, and the cells were manually counted by an experimenter blinded to the experimental groups.

Cerebellar immunofluorescence

Coronal sections (30 μm) of five animals in each group, containing the spinocerebellum and cerebrocerebellum areas were obtained using a cryostat (Leica, Germany). The slices were prepared based on the plates 60 to 62 of the Paxinos and Watson [43] atlas. The slices were then labeled with the following antibodies: anti-glia fibrillary acidic protein-astrocyte marker (GFAP; 1:3000, Sigma-Aldrich), anti-NeuN (neuronal marker; 1:3000, Millipore). The secondary antibodies were rabbit Ig or mouse Ig Alexa Fluor 488 or 555. The sections were washed in PBS and blocked for 30 min with 3% bovine albumin serum (BSA; Sigma-Aldrich) in PBS with 0.3% Triton-X 100 at room temperature. The sections were then incubated overnight with primary antibody at 4°C in PBS, 0.3% Triton X-

100 and 3% BSA. On the following day, the sections were washed in PBS and incubated with the fluorescent antibodies for 2 h at room temperature in a dark chamber, washed in PBS, mounted and cover slipped with anti-fading mounting acidic mount media (DPX). The slides were visualized with a microscope (Nikon TE300; Nikon, Osaka, Japan) coupled to a CCD camera (Quantix 512-Roper Scientific Inc., Princeton Instruments, Princeton, NJ). Images were acquired in BioIP software (Anderson Eng, Delaware, USA).

Oxidative stress parameters

The cerebellum was homogenized in 10 volumes (1:10; w/v) of 20 mM sodium phosphate buffer, plus 140 mM KCl pH 7.4 and centrifuged at 750 *g*, the supernatants were saved and used to determine the oxidative stress parameters.

Reduced glutathione levels (GSH) GSH levels were estimated based on the method described by Browne and Armstrong [48]. Deproteinized samples were mixed to 100 mM sodium phosphate buffer plus 5 mM EDTA. In a dark room, the fluorescent compound (*O*-phthaldialdehyde 1 mg/ml diluted in methanol) was added to the media containing the samples. After 15 min the fluorescence was measured with 420 nm emission and 350 nm excitation. A standard curve was performed using purified GSH. All samples were run in triplicate.

Sulfhydryl content 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB) is reduced by sulfhydryl groups. The product of this reaction is the TNB, which is a yellow compound. Briefly, 15 μ L of sample were added to 275 μ L of phosphate buffer

saline pH 7.4 containing 1 mM EDTA. The reaction was started by the addition of 10 μ L of 10 mM DTNB and incubated for 30 min at room temperature in a dark room. The sulfhydryl content is inversely correlated to oxidative damage to the protein. Results were reported as nmol of TNB per milligram of protein. Absorbance was measured at 412 nm. One blank probe was performed for each sample, whose values were discounted [37]. All samples were run in triplicate.

Single cell gel electrophoresis (comet assay) DNA damage was assessed by alkaline comet assay, as described by Singh and colleagues [49], following general recommendation for comet assay [50, 51]. Cerebellar homogenized samples were suspended in agarose and spread onto a glass microscope slide pre-coated with agarose. Agarose was allowed to set at 4°C for 5 min. Subsequently, the slides were incubated in ice-cold lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10.0, and 1% triton X-100 with 10% DMSO) to clean cell proteins, leaving DNA as 'nucleoids'. Afterwards, the slides were placed on a horizontal electrophoresis system, covered with fresh solution (300 mM NaOH plus 1 mM EDTA, pH > 13) for 20 min at 4°C to allow DNA unwinding and express of alkali-labilesites. The electrophoresis was run for 20 min (25 V; 315 mA; 0.9 V/cm). The slides were then neutralized, washed in bidistilled water and stained with silver staining protocol. After drying at room temperature overnight, gels were analyzed in optical microscope. One hundred cells (50 cells from each of the two replicate slides) were evaluated. Cells were visually scored according to the tail length, receiving scores from 0 (no migration) to 4 (maximal migration) according to tail intensity. Although,

the damage index for cells ranged from 0 (all cells with no migration) to 400 (all cells with maximal migration), all slides were analyzed under blind conditions by two investigators. Mean values of the scores were presented.

Brain-derived neurotrophic factor (BDNF) immunocontent

The mature BDNF protein was estimated using the E-Max ELISA kit (Promega). The assay was performed according to the manufacturer's recommendations and how described by Scherer and colleagues [52].

Western blot analysis

The cerebellum was homogenized in lysis solution (2 mM EDTA, 50 mM Tris-HCl, pH 6.8, plus 4% SDS) the proteins in the samples were then determined, subsequently mixed (v/v) in Laemmli buffer (40% glycerol, 5% mercaptoethanol, 50 mM Tris-HCl, pH 6.8) and then boiled for 3 min. For the assay, samples were loaded (30 µg of protein/lane) in 10% polyacrylamide SDS-PAGE. The proteins were transferred (Trans-blot SD semidry transfer cell; Bio-Rad) to nitrocellulose membranes, for 1 h at 15 V in a transfer buffer (48 mM Trizma, 39 mM glycine, 20% methanol and 0.25% SDS). In the next step, the membrane was washed during 10 min in Tris buffer saline (TBS; 500 mM NaCl, 20 mM Trizma, pH 7.5). The membranes were blocked with TBS plus 5% bovine serum albumin (BSA), for 2 h. Afterwards, the membrane was washed twice during 5 min in T-TBS; TBS plus 0.05% Tween-20. Subsequently, they were incubated overnight at 4°C in a blocking solution plus the following anti-bodies: rabbit anti-AChE (1:1000; Santa

Cruz Biotechnology); rabbit anti-GFAP (1:2000; Alpha Diagnostic International) rabbit anti-capase-3 (1:1000; Sygma); rabbit anti- β -actin (1:1000; Sygma). Then the membranes were washed twice for 5 min with T-TBS and incubated for 2 h in antibody solution containing peroxidase-conjugated anti-rabbit IgG (1:2000; Santa Cruz Biotechnology). In the last step, the membranes were washed twice again for 5 min with T-TBS and twice for 5 min with TBS. They were developed using chemiluminescent ECL kit (Immobilon Western Chemiluminescent HRP Substrate, Millipore) and detected by ImageQuant LAS 4000 (GE Healthcare Life Sciences).

AChE activity assay

The cerebellum was homogenized in ten volumes (1:10; w/v) of 0.1 mM potassium phosphate buffer, pH 7.5 and centrifuged for 10 min at 1.000 g. Hydrolysis rates were measured at acetylthiocholine concentration of 0.8 mM in 300 μ L assay solution with 30 mM phosphate buffer, pH 7.5, and 1.0 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) at 25°C. Supernatant from homogenized tissue was added to the reaction mixture and pre incubated for 3 min. The hydrolysis was monitored by formation of the thiolate dianion of DTNB at 412 nm for 2–3 min (intervals of 30 s) (Ellman et al., 1961), with modifications [52, 54]. All samples were run in triplicate.

Protein determination

The protein amount in the samples was estimated by the method described by Lowry and colleagues [55] for all techniques, with exception of the AChE activity where the Bradford [56] method was used.

Statistical analyses

All data were evaluated by Student's *t* test. Behavioral data were presented as mean \pm S.E.M.; histological and biochemical data were presented as mean \pm S.D. For all tests $p < 0.05$ was assumed as statistically significant. All analyses were performed with the software Statistical Package for the Social Sciences (SPSS; v.20).

Results

First, we investigated the effect of GAL administration on motor coordination assessed by beam walking test. The results showed that GAL does not cause changes in motor coordination 1 h [$t(21) = 0.2642$; $p > 0.05$] after injection. However, rats that received GAL showed significant motor deficit 24 h [$t(21) = 2.461$; $p < 0.05$] after GAL administration (Fig. 1).

Considering the results of motor coordination at 24 h after GAL injection, we decided to evaluate histological and biochemical parameters in cerebellum of rats at 24 h after GAL injection. The number of cells was counted in the molecular and granular layers of the spinocerebellum and cerebrocerebellum 24 h after GAL injection. Fig. 2A and B show that GAL reduced the number of the cells in the molecular [$t(10) = 2.328$; $p < 0.05$] and granular [$t(10) = 2.770$; $p < 0.05$] layers of the spinocerebellum. However, the number of cells were decreased only in the molecular [$t(10) = 2.923$; $p < 0.05$] layer of the cerebrocerebellum (Fig. 2C and D).

Subsequently, we performed immunofluorescence analysis of the spinocerebellum and cerebrocerebellum 24 h after GAL injection, in order to verify which cell type was lost. The slices were co-labeled for immunofluorescence with neuron-specific nuclear antigen (NeuN) and antibody against Glial Fibrillary Acid Protein (GFAP) and analyzed using a fluorescence microscopy. We observed a reduction in GFAP and NeuN-immunostaining in the granular layer of spinocerebellum as compared with control (Fig. 3). In the granular layer of the cerebrocerebellum, GAL injection decreased only NeuN-immunostaining with unaltered GFAP-immunostaining, suggesting neuronal loss in this cerebellar area (Fig. 4). We also verified the GFAP immunocontent in the whole cerebellum using the western blot technique, and the result showed no alteration in the GFAP immunocontent (*data not shown*).

In order to verify the type of cell death induced by injection of GAL, we carried out immunoblotting assay against active caspase-3, and measured BDNF levels, since caspase-3 is a central protein in caspase dependent apoptosis and BDNF is an important antiapoptotic factor. GAL increased the immunocontent of active caspase-3 [$t(10) = 2.834$; $p < 0.01$] and decreased BDNF levels [$t(10) = 2.990$; $p < 0.05$] at 24 h after GAL injection. These results are compatible with down-regulated survival mechanisms observed in the cerebellum of treated animals.

AChE is found up-regulated in several brain diseases. It controls the end of cholinergic transmission, and its activity and/or immunocontent is upregulated during and after apoptosis. Fig. 6A shows that GAL injection increased AChE activity

[$t(12) = 3.590$; $p < 0.01$] and decreased its immunocontent [$t(12) = 2.340$; $p < 0.05$] in cerebellum of rats (Fig. 6B).

GSH depletion can facilitate or directly induce cell apoptosis, and also plays an important role repairing protein damage. In this context, we found that GAL decreased GSH levels [$t(12) = 2.304$; $p < 0.05$] in the cerebellum of GAL-treated-rats (Fig. 7A). Furthermore, the results of sulfhydryl content showed that GAL decreases the thiol content [$t(12) = 3.347$; $p < 0.01$], suggesting protein damage (Fig. 7B). Also, we assessed DNA damage by alkaline comet assay, and Fig. 7C shows DNA damage [$t(10) = 45.81$; $p < 0.001$] in the rat cerebellum after GAL injection.

Discussion

In the present study, we investigated the influence of a single intracerebroventricular injection of GAL on motor coordination, as well as histological and biochemical parameters in the cerebellum of Wistar rats. We found that GAL decreased motor coordination 24 h after injection. In the spinocerebellum, GAL provoked cell loss in the molecular and granular layers, in which neuronal and astroglial cell loss was observed. In the cerebocerebellum, GAL decreased the cell number in the molecular layer, and immunofluorescence analyses indicated neuronal loss. Thus, the elucidation of the biochemical alterations of GAL-induced neurotoxicity provides new evidences to the neurotoxic mechanisms of this metabolite. In this context, GAL injection provoked enhancement of active caspase-3 immunocontent, decreased BDNF levels, increased AChE activity,

decreased AChE immunocontent, depletion of GSH and sulfhydryl and increased DNA damage.

The beam walking test allows to investigate fine motor coordination and balance in rodents, motor deficit may be induced by brain injury or by genetic and/or pharmacological manipulation [57, 58]. The cerebellum has a fundamental role in the timing and sensory acquisition [59]. Within this perspective, cerebellar damage may be responsible for several motor disturbances such as: ataxia, speech and tremor [59, 60]. Corroborating with findings our results show that the intracerebroventricular GAL injection provokes motor impairments in rats, which may be associated with the phenotype developed in classical galactosemia.

Cerebellar histological parameters were evaluated, in an attempt to better understand the consequences of intracerebroventricular GAL administration on cellular level. We counted the number of cells in the molecular and granular layers of the spinocerebellum and cerebrocerebellum. Results showed loss of cells in the molecular and granular layers of the spinocerebellum, as well as cell loss in the molecular layer of the cerebrocerebellum. It is important to remain that the spinocerebellum processes the sensory motor inputs; while the cerebrocerebellum receives input from the motor cortex and is also involved in high cognitive functions [61].

The immunofluorescence was performed to investigate the spatial influence of the GAL injection on neurons and astrocytes in the spinocerebellum and cerebrocerebellum. Results showed neuronal loss in the granular layers of the

spinocerebellum and cerebrotocerebellum, as well as astroglial loss in the spinocerebellum. These results corroborate with the findings of Crome [21], who described spared granular layer in the cerebellum of a patient, and with the results of diffuse cerebellar atrophy in classical galactosemia patients [19, 62]. Neuronal circuits are important to execute function such as motor [63]. Astrocytes are essential to neuronal survive and synaptic activity. Thus, astrocyte damage may lead to pathologies [64], considering that astrocyte depletion seems to be correlated with neuronal loss [65]. The results of histology and immunohistochemistry suggest that the behavioral change observed is due to cell loss in key cerebellar areas responsible for movement coordination. Our results indicate that the spinocerebellum was more vulnerable to GAL toxicity since astrocyte and neurons were loss in this area.

Apoptosis may be involved in neurodegenerative process, and a wide range of internal and/or external stimuli trigger this cell death process. Caspase dependent is the most common and well known apoptosis type [25]. Caspase-3 has a central role in propagate intrinsic and extrinsic apoptotic signals [25]. Neurotrophins may protect neuronal cells from injury, BDNF plays a key role by inhibiting caspases activation [31]. BDNF depletion in motor structures like the cerebellum has been linked with several motor diseases, due to loss of neuronal survival and/or function [29]. Results showed increased cleaved caspase-3 and reduced BDNF levels, which is suggesting that the neuronal and astroglial losses provoked by GAL injection are at least in part due to apoptosis pathway.

Impairment in the cerebellar cholinergic system has been implicated in several motor diseases, due the involvement of the cholinergic system in movement control [66]. Acetylcholine plays a key role in cell survival, and it has been associated with neurodegenerative diseases [34, 36]. An important finding that gives support is that cells with increased AChE activity can easily switch to an apoptotic state by caspases cleavage [67]. Another important fact of its involvement with cellular death processes is that AChE inhibitors in pathological state slow down neurodegeneration by increasing BDNF levels [35]. In the present study, we found increase in AChE activity and decrease of its immunocontent in cerebellum. We cannot precisely establish the mechanisms that GAL provoked these alterations; the decreased immunocontent may be an attempt to restore the normal AChE activity. Although, our results support that the alterations on AChE activity may be involved in the apoptotic process elicited by GAL.

GSH is a well-known abundant non-enzymatic endogenous antioxidant, and its depletion is linked with apoptosis and damage to biomolecules [38, 40, 68]. Results showed GSH depletion in the rat cerebellum after intracerebroventricular GAL injection, and this data corroborate that GAL injection leads to apoptosis in the cerebellum. Studies showed that low levels of GSH, which may be caused by genetic disorders or increased levels of free radicals, seem to be related to cerebellar lesions that may contribute to motor deficits [16, 69, 70]. Results also showed that GAL injection provoked protein and DNA damage in the rat cerebellum, probably by GSH depletion. Such damages may contribute to cell fate decision for apoptosis observed in the present report [41].

Patients with classical galactosemia usually develop coordination-related impairments [2, 7, 8], ataxia and tremor are the most frequent observed complications [7]. GAL is found accumulated at millimolar concentrations in the brain of patients [46, 71]. The aging model applied in mice and rats that utilizes chronic GAL administration has been showed several brain alterations provoked by GAL [72, 73]. In this model, the authors described motor impairment, suggesting to be due to cellular and biochemical alterations in the cerebellum [73]. Considering that in our study the concentration of GAL was similar to those found in brain of classical galactosemia patients, our findings are adding another piece to the puzzle of GAL neurotoxicity in classical galactosemia.

In summary, in the present study, we showed that GAL provokes coordination impairment and cell loss in key cerebellar regions responsible for movement tuning. We also demonstrated that, at least part, the cell loss is mediated by apoptosis, which may be deeply influenced by the diminished BDNF and GSH levels, increased AChE activity and damage to biomolecules. Our findings reinforce the importance of GAL restriction as treatment, and may contribute to the understanding of the neurotoxicity mechanisms behind the neuropathology of classical galactosemia.

Conflict of interest

The authors declare to have no conflict of interest.

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References

1. Calderon FRO, Phansalkar AR, Crockett DK, Miller M, Mao R (2007) Mutation database for the galactose-1-phosphate uridylyltransferase (GALT) gene. *Hum Mutat* 28:939–943. doi: 10.1002/humu.20544
2. Fridovich-Keil JL, Walter J (2008) Galactosemia. In: Valle D, Beaudet A, Vogelstein B, Kinzler K, Antonarakis S, Ballabio A (eds) online *Metab. Mol. bases Inherit. Dis.* McGrawHill, pp 1–74
3. Marinou K, Tsakiris S, Tsopanakis C, Schulpis KH, Behrakis P (2005) Suckling rat brain regional distribution of acetylcholinesterase activity in galactosaemia in vitro. *Metab Brain Dis* 20:227–236. doi: 10.1007/s11011-005-7210-3
4. Jumbo-Lucioni PP, Garber K, Kiel J, Baric I, Berry GT, Bosch A, Burlina A, Chiesa A, Pico MLC, Estrada SC, Henderson H, Leslie N, Longo N, Morris AAM, Ramirez-Farias C, Scheweitzer-Krantz S, Silao CLT, Vela-Amieva M, Waisbren S, Fridovich-Keil JL (2012) Diversity of approaches to classic galactosemia around the world: a comparison of diagnosis, intervention, and outcomes. *J Inherit Metab Dis* 35:1037–1049. doi: 10.1007/s10545-012-9477-y
5. Waggoner DD, Buist NRM, Donnell GN (1990) Long-term prognosis in galactosaemia: Results of a survey of 350 cases. *J Inherit Metab Dis* 13:802–818. doi: 10.1007/BF01800204
6. Waisbren SE, Potter NL, Gordon CM, Green RC, Greenstein P, Gubbels CS, Rubio-Gozalbo E, Schomer D, Welt C, Anastasoie V, D'Anna K, Gentile J, Guo CY, Hecht L, Jackson R, Jansma BM, Li Y, Lip V, Miller DT, Murray M, Power L, Quinn N, Rohr F, Shen Y, Skinder-Meredith A, Timmers I, Tunick R, Wessel A, Wu BL, Levy H, Elsas L, Berry GT (2012) The adult galactosemic phenotype. *J Inherit Metab Dis* 35:279–286. doi: 10.1007/s10545-011-9372-y
7. Rubio-Agusti I, Carecchio M, Bhatia KP, Kojovic M, Parees I, Chandrashekar HS, Footitt EJ, Burke D, Edwards MJ, Lachmann RHL, Murphy E (2013) Movement Disorders in Adult Patients With Classical Galactosemia. *Mov Disord* 28:804–810. doi: 10.1002/mds.25348
8. Potter NL, Nievergelt Y, Shriberg LD (2013) Motor and Speech Disorders in Classic Galactosemia. *JIMD Rep* 11:31–41. doi: 10.1007/8904_2013_219
9. Ridel KR, Leslie ND, Gilbert DL (2005) An updated review of the long-term neurological effects of galactosemia. *Pediatr Neurol* 33:153–161. doi: 10.1016/j.pediatrneurol.2005.02.015
10. Jumbo-Lucioni PP, Hopson ML, Hang D, Liang Y, Jones DP, Fridovich-Keil JL (2013) Oxidative stress contributes to outcome severity in a *Drosophila*

- melanogaster model of classic galactosemia. *Dis Model Mech* 6:84–94. doi: 10.1242/dmm.010207
11. Schulpis KH, Michelakakis H, Tsakiris T, Tsakiris S (2005) The effect of diet on total antioxidant status, erythrocyte membrane Na⁺,K⁺-ATPase and Mg²⁺-ATPase activities in patients with classical galactosaemia. *Clin Nutr* 24:151–157. doi: 10.1016/j.clnu.2004.09.001
 12. Schulpis KH, Papassotiriou I, Tsakiris S (2006) 8-hydroxy-2-desoxyguanosine serum concentrations as a marker of DNA damage in patients with classical galactosaemia. *Acta Paediatr* 95:164–169. doi: 10.1080/08035250500297810
 13. Slepak TI, Tang M, Slepak VZ, Lai K (2007) Involvement of endoplasmic reticulum stress in a novel Classic Galactosemia model. *Mol Genet Metab* 92:78–87. doi: 10.1016/j.ymgme.2007.06.005
 14. Coss KP, Treacy EP, Cotter EJ, Knerr I, Murray DW, Shin YS, Doran PP (2014) Systemic gene dysregulation in classical Galactosaemia: Is there a central mechanism? *Mol Genet Metab* 113:177–187. doi: 10.1016/j.ymgme.2014.08.004
 15. Schmahmann JD (2004) Disorders of the cerebellum: ataxia, dysmetria of thought, and the cerebellar cognitive affective syndrome. *J Neuropsychiatry Clin Neurosci* 16:367–378. doi: 10.1176/appi.neuropsych.16.3.367
 16. Doss S, Rinnenthal JL, Schmitz-Hübsch T, Brandt AU, Papazoglou S, Lux S, Maul S, Würfel J, Endres M, Klockgether T, Minnerop M, Paul F (2015) Cerebellar neurochemical alterations in spinocerebellar ataxia type 14 appear to include glutathione deficiency. *J Neurol* 262:1927–1935. doi: 10.1007/s00415-015-7788-2
 17. Ramnani N (2006) The primate cortico-cerebellar system: anatomy and function. *Nat Rev Neurosci* 7:511–522. doi: 10.1038/nrn1953
 18. Stoodley CJ, Valera EM, Schmahmann JD (2012) Functional topography of the cerebellum for motor and cognitive tasks: An fMRI study. *Neuroimage* 59:1560–1570. doi: 10.1016/j.neuroimage.2011.08.065
 19. Kaufman FR, McBride-Chang C, Manis FR, Wolff JA, Nelson MD (1995) Cognitive functioning, neurologic status and brain imaging in classical galactosemia. *Eur J Pediatr* 154:S2–5.
 20. Böhles H, Wenzel D, Shin YS (1986) Progressive cerebellar and extrapyramidal motor disturbances in galactosaemic twins. *Eur J Pediatr* 145:413–417. doi: 10.1007/BF00439251
 21. Crome L (1962) A case of galactosaemia with the pathological and neuropathological findings. *Arch Dis Child* 37:415–421.
 22. Heimfarth L, Loureiro SO, Dutra MF, Petenuzzo L, de Lima BO, Fernandes CG, da Rocha JBT, Pessoa-Pureur R (2013) Disrupted cytoskeletal homeostasis, astrogliosis and apoptotic cell death in the cerebellum of preweaning rats injected with diphenyl ditelluride. *Neurotoxicology* 34:175–188. doi: 10.1016/j.neuro.2012.10.015
 23. Gorman AM (2008) Neuronal cell death in neurodegenerative diseases: recurring themes around protein handling. *J Cell Mol Med* 12:2263–2280. doi: 10.1111/j.1582-4934.2008.00402.x
 24. Clarke PG. (2002) Apoptosis: from morphological types of cell death to

- interacting pathways. *Trends Pharmacol Sci* 23:308–309. doi: 10.1016/S0165-6147(02)02041-2
25. Hongmei Z (2012) Extrinsic and Intrinsic Apoptosis Signal Pathway Review. In: *Apoptosis Med. InTech*, pp 3–22
 26. Cui X, Zuo P, Zhang Q, Li X, Hu Y, Long J, Packer L, Liu J (2006) Chronic systemic D-galactose exposure induces memory loss, neurodegeneration, and oxidative damage in mice: Protective effects of R- α -lipoic acid. *J Neurosci Res* 84:647–654. doi: 10.1002/jnr.20899
 27. Elmore S (2007) Apoptosis: a review of programmed cell death. *Toxicol Pathol* 35:495–516. doi: 10.1080/01926230701320337
 28. Lamkanfi M (2002) Alice in caspase land. A phylogenetic analysis of caspases from worm to man. *Cell Death Differ* 9:358–361. doi: 10.1038/sj/cdd/4400989
 29. He YY, Zhang XY, Yung WH, Zhu JN, Wang JJ (2013) Role of BDNF in central motor structures and motor diseases. *Mol Neurobiol* 48:783–793. doi: 10.1007/s12035-013-8466-y
 30. Lu B (2003) Pro-Region of Neurotrophins. *Neuron* 39:735–738. doi: 10.1016/S0896-6273(03)00538-5
 31. Kim DH, Zhao X (2005) BDNF Protects Neurons Following Injury by Modulation of Caspase Activity. *Neurocrit Care* 3:71–76. doi: 10.1385/NCC:3:1:071
 32. Zafra F, Lindholm D, Castrén E, Hartikka J, Thoenen H (1992) Regulation of brain-derived neurotrophic factor and nerve growth factor mRNA in primary cultures of hippocampal neurons and astrocytes. *J Neurosci* 12:4793–4799.
 33. Knipper M, Penha Berzaghi M, Blöchl A, Breer H, Thoenen H, Lindholm D (1994) Positive Feedback between Acetylcholine and the Neurotrophins Nerve Growth Factor and Brain-derived Neurotrophic Factor in the Rat Hippocampus. *Eur J Neurosci* 6:668–671. doi: 10.1111/j.1460-9568.1994.tb00312.x
 34. Ferreira AGK, Scherer EB, da Cunha MJ, Machado FR, da Cunha AA, Graeff JS, Netto CA, Wyse ATS (2011) Physical Exercise Reverses Cognitive Impairment in Rats Subjected to Experimental Hyperprolinemia. *Neurochem Res* 36:2306–2315. doi: 10.1007/s11064-011-0555-6
 35. Bendix I, Serdar M, Herz J, von Haefen C, Nasser F, Rohrer B, Endesfelder S, Felderhoff-Mueser U, Spies CD, Sifringer M (2014) Inhibition of acetylcholinesterase modulates NMDA receptor antagonist mediated alterations in the developing brain. *Int J Mol Sci* 15:3784–3798. doi: 10.3390/ijms15033784
 36. Resende RR, Adhikari A (2009) Cholinergic receptor pathways involved in apoptosis, cell proliferation and neuronal differentiation. *Cell Commun Signal* 7:20. doi: 10.1186/1478-811X-7-20
 37. Aksenov MY, Markesbery WR (2001) Changes in thiol content and expression of glutathione redox system genes in the hippocampus and cerebellum in Alzheimer's disease. *Neurosci Lett* 302:141–145. doi: 10.1016/S0304-3940(01)01636-6
 38. Halliwell B (2011) Free radicals and antioxidants - Quo vadis? *Trends Pharmacol Sci* 32:125–130. doi: 10.1016/j.tips.2010.12.002

39. Franco R, Cidlowski JA (2009) Apoptosis and glutathione: beyond an antioxidant. *Cell Death Differ* 16:1303–1314. doi: 10.1038/cdd.2009.107
40. Circu ML, Aw TY (2012) Glutathione and modulation of cell apoptosis. *Biochim Biophys Acta* 1823:1767–1777. doi: 10.1016/j.bbamcr.2012.06.019
41. Hamdi Y, Kaddour H, Vaudry D, Leprince J, Zarrouk A, Hammami M, Vaudry H, Tonon MC, Amri M, Masmoudi-Kouki O (2015) Octadecaneuropeptide ODN prevents hydrogen peroxide-induced oxidative damage of biomolecules in cultured rat astrocytes. *Peptides* 71:56–65. doi: 10.1016/j.peptides.2015.06.010
42. Delwing D, Delwing D, Bavaresco CS, Wyse ATS (2008) Protective effect of nitric oxide synthase inhibition or antioxidants on brain oxidative damage caused by intracerebroventricular arginine administration. *Brain Res* 1193:120–127. doi: 10.1016/j.brainres.2007.11.052
43. Paxinos G, Watson C (1986) *The Rat Brain in Stereotaxic Coordinates*. London
44. Tsakiris S, Marinou K, Schulpis KH (2002) The in vitro Effects of Galactose and its Derivatives on Rat Brain Mg²⁺-ATPase Activity. *Pharmacol Toxicol* 91:254–257. doi: 10.1034/j.1600-0773.2002.910506.x
45. Tsakiris S, Carageorgiou H, Schulpis KH (2005) The protective effect of L-cysteine and glutathione on the adult and aged rat brain (Na⁺,K⁺)-ATPase and Mg²⁺-ATPase activities in galactosemia in vitro. *Metab Brain Dis* 20:87–95. doi: 10.1007/s11011-005-2480-3
46. Berry GT (2011) Is prenatal myo-inositol deficiency a mechanism of CNS injury in galactosemia? *J Inherit Metab Dis* 34:345–355. doi: 10.1007/s10545-010-9260-x
47. Lotan D, Benhar I, Alvarez K, Mascaro-Blanco A, Brimberg L, Frenkel D, Cunningham MW, Joel D (2014) Behavioral and neural effects of intra-striatal infusion of anti-streptococcal antibodies in rats. *Brain Behav Immun* 38:249–262. doi: 10.1016/j.bbi.2014.02.009
48. Browne RW, Armstrong D (1998) Reduced glutathione and glutathione disulfide. *Methods Mol Biol* 108:347–352. doi: 10.1385/0-89603-472-0:347
49. Singh NP, McCoy MT, Tice RR, Schneider EL (1988) A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp Cell Res* 175:184–191.
50. Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A, Kobayashi H, Miyamae Y, Rojas E, Ryu JC, Sasaki YF (2000) Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. *Environ Mol Mutagen* 35:206–221.
51. Hartmann A, Agurell E, Beevers C, Brendler-Schwaab S, Burlinson B, Clay P, Collins A, Smith A, Speit G, Thybaud V, Tice RR, 4th International Comet Assay Workshop (2003) Recommendations for conducting the in vivo alkaline Comet assay. *Mutagenesis* 18:45–51.
52. Scherer EBS, da Cunha MJ, Matté C, Schmitz F, Netto CA, Wyse ATS (2010) Methylphenidate affects memory, brain-derived neurotrophic factor immunoccontent and brain acetylcholinesterase activity in the rat. *Neurobiol Learn Mem* 94:247–253. doi: 10.1016/j.nlm.2010.06.002
53. Ellman GL, Courtney KD, Andres V, Featherstone RM (1961) A new and

- rapid colorimetric determination of acetylcholinesterase activity. *Biochem Pharmacol* 7:88–95. doi: 10.1016/0006-2952(61)90145-9
54. Delwing D, Chiarani F, Delwing D, Bavaresco CS, Wannmacher CMD, Wajner M, Wyse ATS (2003) Proline reduces acetylcholinesterase activity in cerebral cortex of rats. *Metab Brain Dis* 18:79–86.
 55. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* 193:265–275.
 56. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254. doi: 10.1016/0003-2697(76)90527-3
 57. Luong TN, Carlisle HJ, Southwell A, Patterson PH (2011) Assessment of Motor Balance and Coordination in Mice using the Balance Beam. *J Vis Exp* 2376. doi: 10.3791/2376
 58. Brooks SP, Dunnett SB (2009) Tests to assess motor phenotype in mice: a user's guide. *Nat Rev Neurosci* 10:519–529. doi: 10.1038/nrn2652
 59. Manto M, Bower JM, Conforto AB, Delgado-García JM, da Guarda SNF, Gerwig M, Habas C, Hagura N, Ivry RB, Mariën P, Molinari M, Naito E, Nowak DA, Taib NOB, Pelisson D, Tesche CD, Tilikete C, Timmann D (2012) Consensus Paper: Roles of the Cerebellum in Motor Control—The Diversity of Ideas on Cerebellar Involvement in Movement. *The Cerebellum* 11:457–487. doi: 10.1007/s12311-011-0331-9
 60. Apps R, Garwicz M (2005) Anatomical and physiological foundations of cerebellar information processing. *Nat Rev Neurosci* 6:297–311. doi: 10.1038/nrn1646
 61. Kandel ER, Schwartz JH, Jessell TM, Siegelbaum SA, Hudspetf AJ (2013) *Principles of Neural Science.*, 5th ed. McGraw-Hill, New York
 62. Nelson MD, Wolff JA, Cross CA, Donnell GN, Kaufman FR (1992) Galactosemia: evaluation with MR imaging. *Radiology* 184:255–261. doi: 10.1148/radiology.184.1.1319076
 63. Yuste R (2015) From the neuron doctrine to neural networks. *Nat Rev Neurosci* 16:487–497. doi: 10.1038/nrn3962
 64. Gundersen V, Storm-Mathisen J, Bergersen LH (2015) Neuroglial Transmission. *Physiol Rev* 95:695–726. doi: 10.1152/physrev.00024.2014
 65. Schreiner B, Romanelli E, Liberski P, Ingold-Heppner B, Sobottka-Brillout B, Hartwig T, Chandrasekar V, Johannssen H, Zeilhofer HU, Aguzzi A, Heppner F, Kerschensteiner M, Becher B (2015) Astrocyte Depletion Impairs Redox Homeostasis and Triggers Neuronal Loss in the Adult CNS. *Cell Rep* 12:1377–1384. doi: 10.1016/j.celrep.2015.07.051
 66. Quik M, Zhang D, Perez XA, Bordia T (2014) Role for the nicotinic cholinergic system in movement disorders; therapeutic implications. *Pharmacol Ther* 144:50–59. doi: 10.1016/j.pharmthera.2014.05.004
 67. Zhang XJ, Greenberg DS (2012) Acetylcholinesterase Involvement in Apoptosis. *Front Mol Neurosci* 5:1–6. doi: 10.3389/fnmol.2012.00040
 68. Aoyama K, Nakaki T (2015) Glutathione in Cellular Redox Homeostasis: Association with the Excitatory Amino Acid Carrier 1 (EAAC1). *Molecules* 20:8742–8758. doi: 10.3390/molecules20058742
 69. Ristoff E, Larsson A (2007) Inborn errors in the metabolism of glutathione.

- Orphanet J Rare Dis 2:16. doi: 10.1186/1750-1172-2-16
70. Franco JL, Teixeira A, Meotti FC, Ribas CM, Stringari J, Garcia Pomblum SC, Moro ÂM, Bohrer D, Bairros A V, Dafre AL, Santos ARS, Farina M (2006) Cerebellar thiol status and motor deficit after lactational exposure to methylmercury. *Environ Res* 102:22–28. doi: 10.1016/j.envres.2006.02.003
 71. Tsakiris S, Schulpis KH (2000) The effect of galactose metabolic disorders on rat brain acetylcholinesterase activity. *Zeitschrift Fur Naturforsch C* 55:852–855.
 72. Parameshwaran K, Irwin MH, Steliou K, Pinkert CA (2010) D-Galactose Effectiveness in Modeling Aging and Therapeutic Antioxidant Treatment in Mice. *Rejuvenation Res* 13:729–735. doi: 10.1089/rej.2010.1020
 73. Banji D, Banji OJF, Dasaroju S, Kranthi KCH (2013) Curcumin and piperine abrogate lipid and protein oxidation induced by D-galactose in rat brain. *Brain Res* 1515:1–11. doi: 10.1016/j.brainres.2013.03.023

Legend of figures

Fig. 1 Effect of intracerebroventricular injection of galactose on motor coordination. Beam walking test was performed 1 h and 24 h after galactose injection. Data are expressed as mean \pm S.E.M. for 10-13 animals in each group. * $p < 0.05$; Different from the control group (Student's *t* test).

Fig. 2 Effect of intracerebroventricular injection of galactose on the number of cells in the molecular and granular layers of the spinocerebellum (A and B) and cerebellocerebellum (C and D) 24 h after galactose injection. The cell number was estimated by manually cell counting in 10 fields ($14400 \mu\text{m}^2$) per animal in each layer. Data are expressed as mean \pm S.D. for 6 animals in each group. * $p < 0.05$. Different from the control group (Student's *t* test). Representative images of 6 animals. ML = molecular layer; GL = granular layer. Bar scale = $50 \mu\text{m}$ (magnification: 20x).

Fig. 3 Effect of intracerebroventricular injection of galactose on immunohistochemistry for GFAP, NeuN and merged in the spinocerebellum of rats 24 h after galactose injection. The panel shows decreased NeuN and GFAP staining, indicating neuronal and astroglial cell loss in the spinocerebellum 24 h after galactose injection. Representative images of 5 animals. ML = molecular layer; GL = granular layer. Bar scale = 30 μm (magnification: 40x); 50 μm (magnification: 20x).

Fig. 4 Effect of intracerebroventricular injection of galactose on immunohistochemistry for GFAP, NeuN and merged in the cerebrocerebellum of rats 24 h after galactose injection. The panel shows decreased NeuN staining, indicating neuronal cell loss in the cerebrocerebellum 24 h after galactose injection. Representative images of 5 animals. ML = molecular layer; GL = granular layer. Bar scale = 30 μm (magnification: 40x); 50 μm (magnification: 20x).

Fig. 5 Effect of intracerebroventricular injection of galactose on active caspase-3 immunocontent (A) and BDNF immunocontent (B) in the cerebellum of rats 24 h after galactose injection. Data are expressed as mean \pm S.D. for 6-7 animals in each group. * $p < 0.05$; ** $p < 0.01$; Different from the control group (Student's *t* test).

Fig. 6 Effect of intracerebroventricular injection of galactose on acetylcholinesterase activity (A) and acetylcholinesterase immunocontent (B) in the cerebellum of rats 24 h after galactose injection. Data are expressed as mean \pm S.D. for 6 animals in each group. * $p < 0.05$; ** $p < 0.01$; Different from the control group (Student's *t* test).

Fig. 7 Effect of intracerebroventricular injection of galactose on glutathione levels (A), sulfhydryl levels (B) and DNA damage (C) in the cerebellum of rats 24 h after galactose injection. Data are expressed as mean \pm S.D. for 6-7 animals in each group. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; Different from the control group (Student's *t* test).

Figures

Fig. 1.

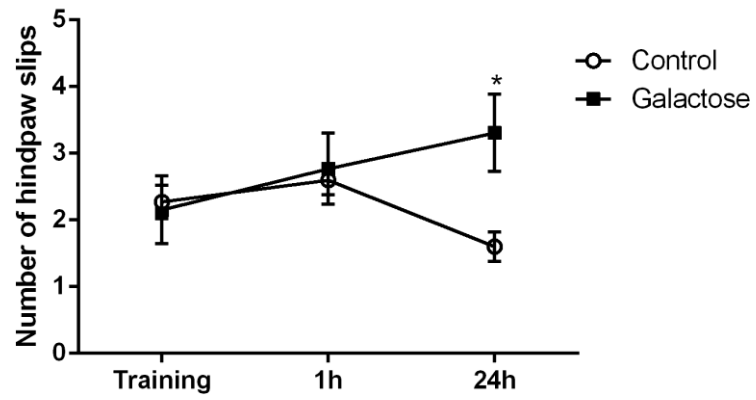


Fig. 2.

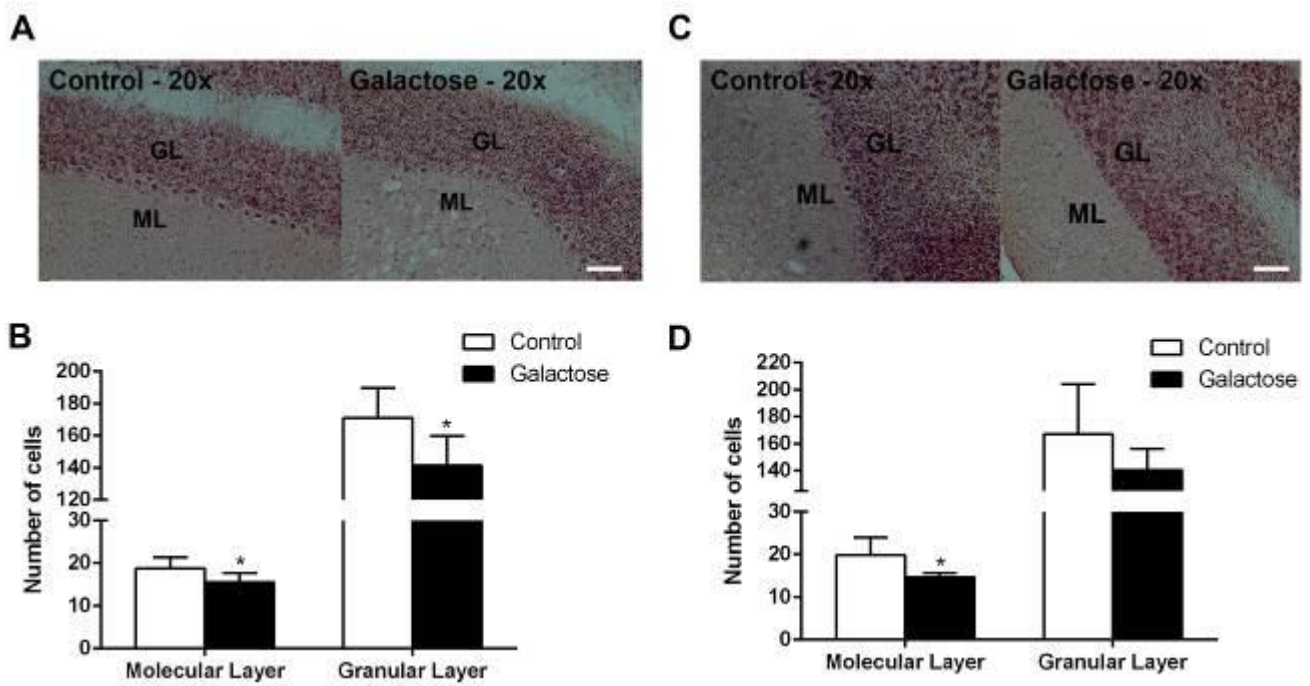


Fig. 3.

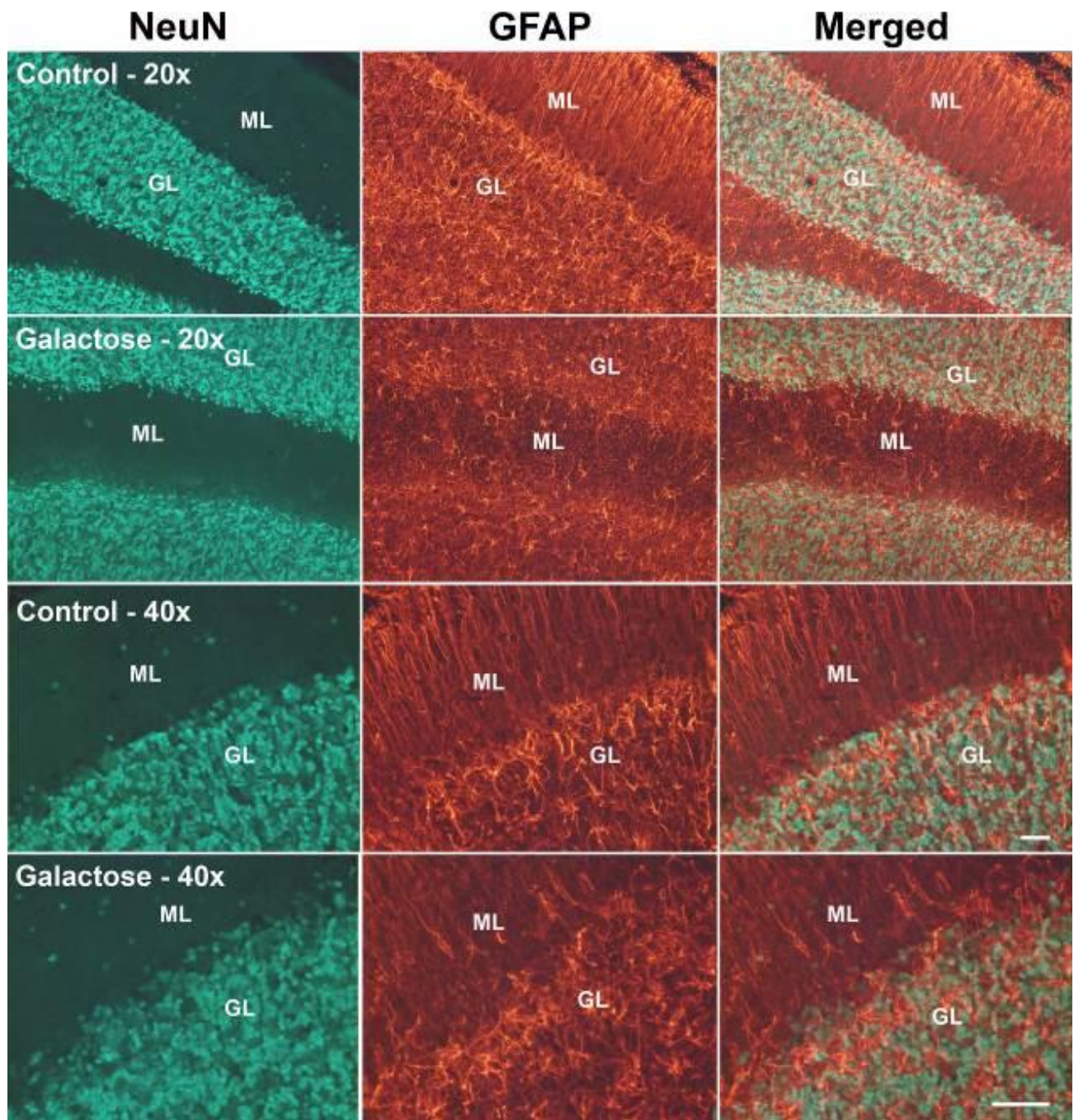


Fig. 4.

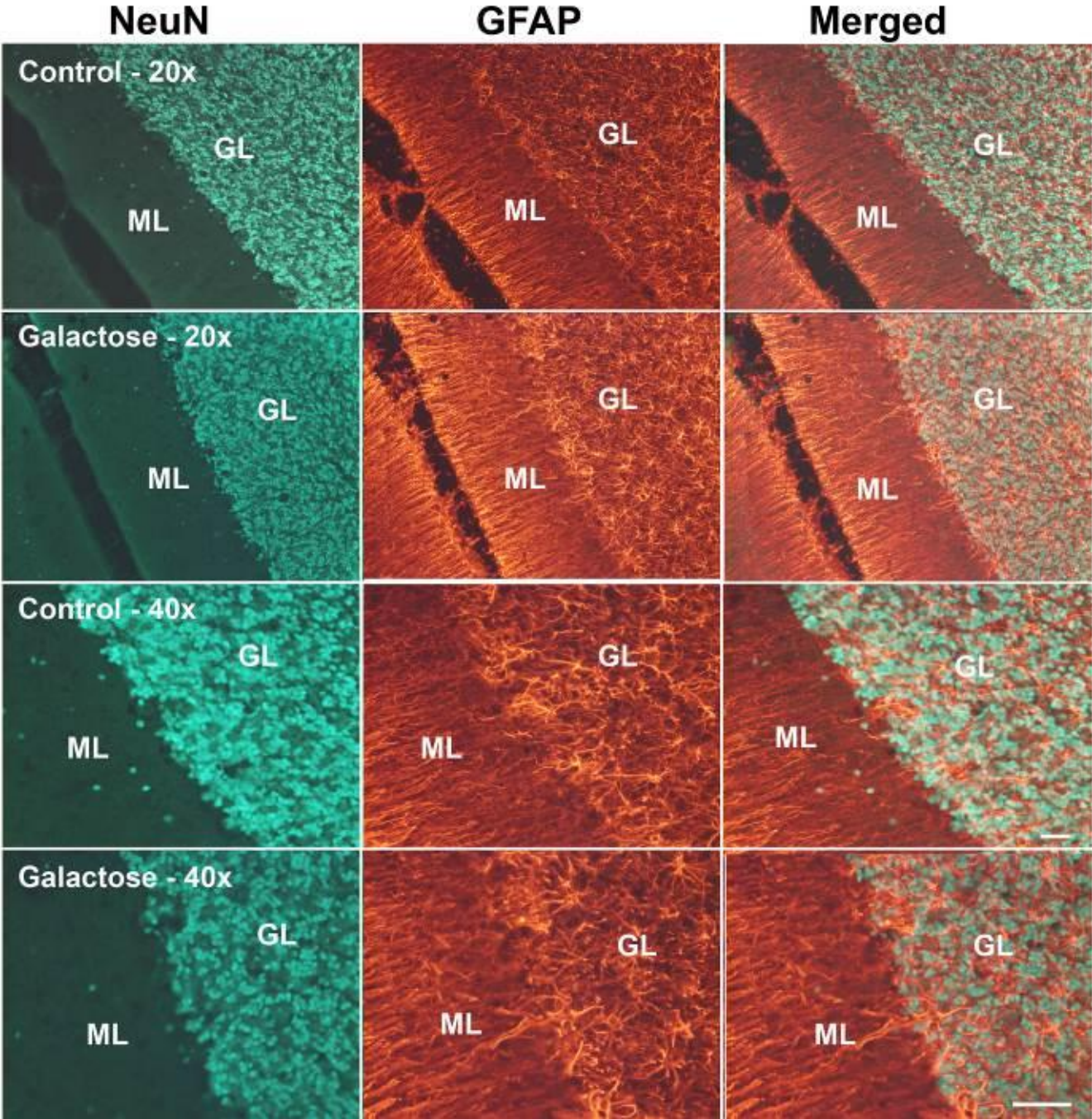


Fig. 5.

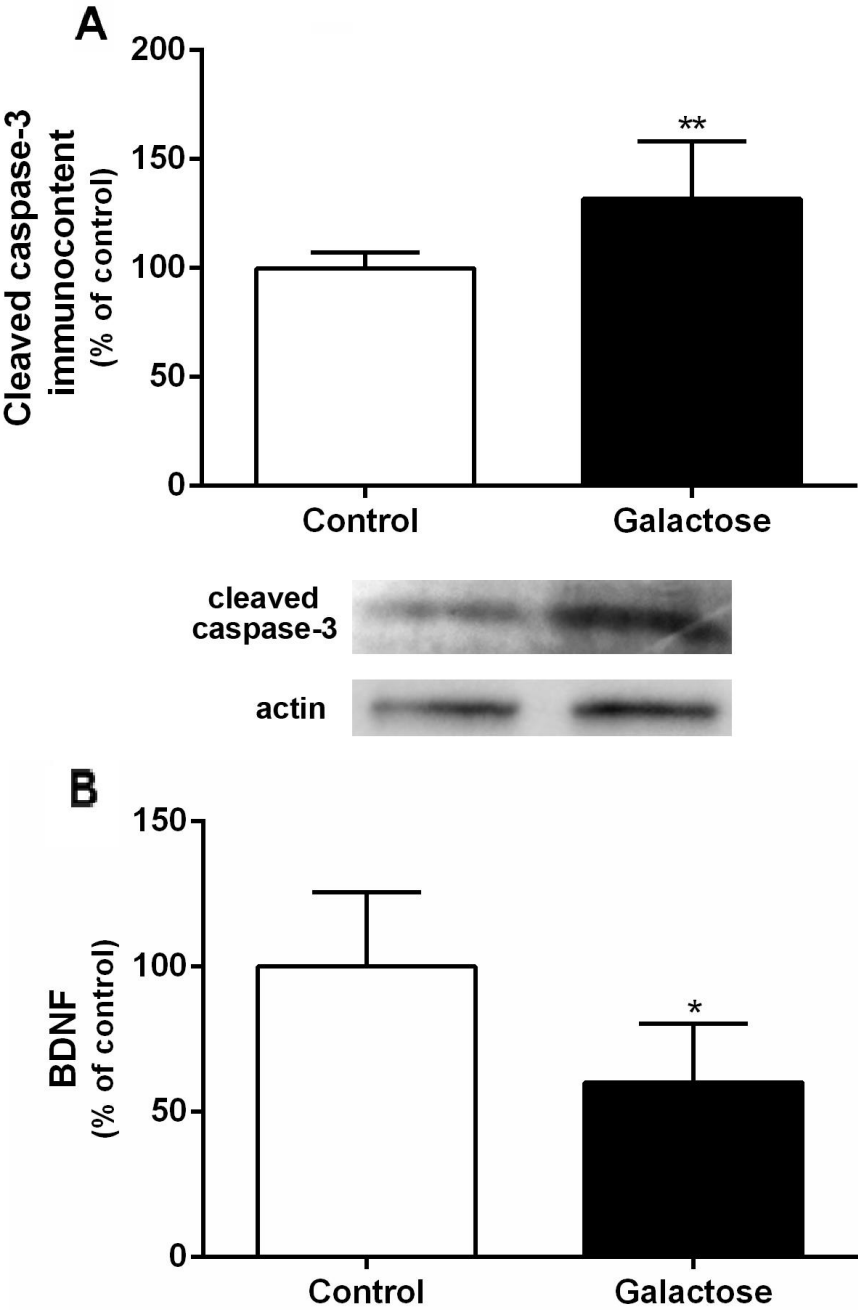


Fig. 6.

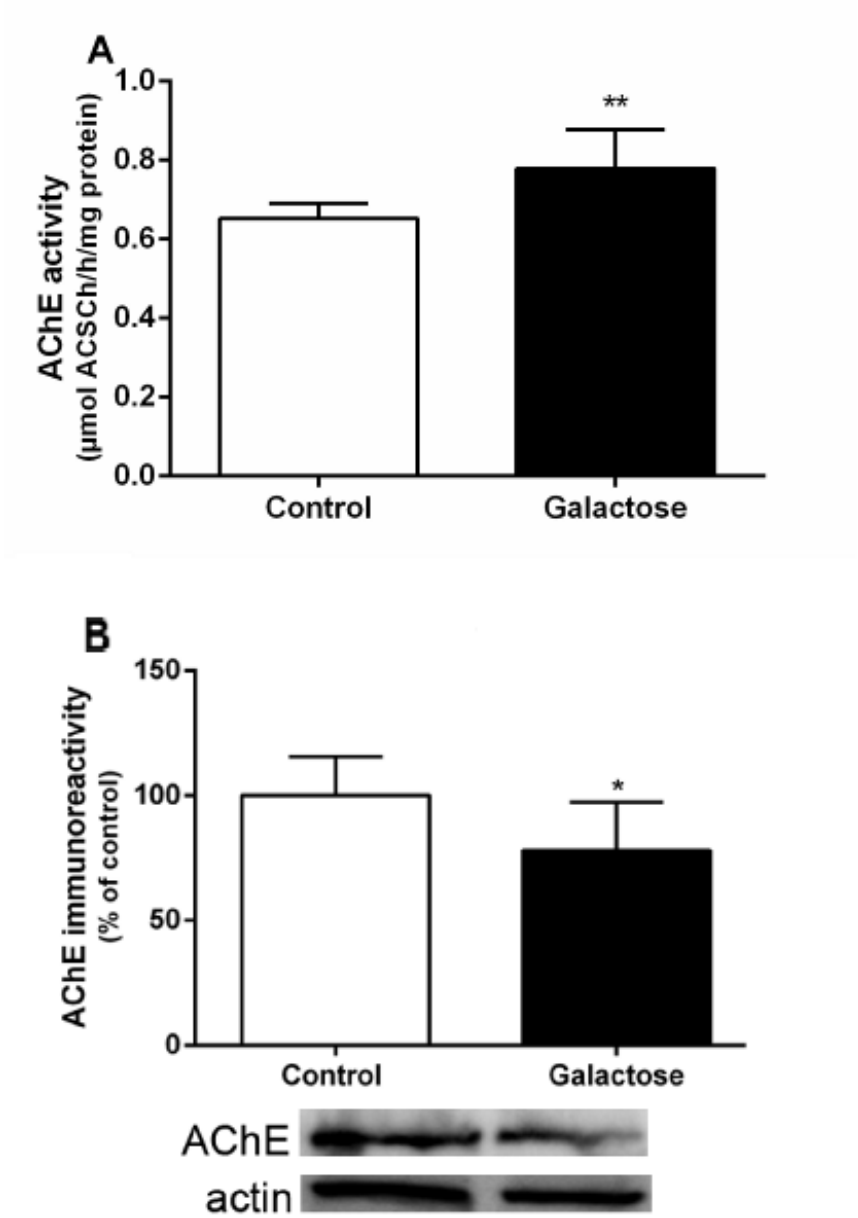
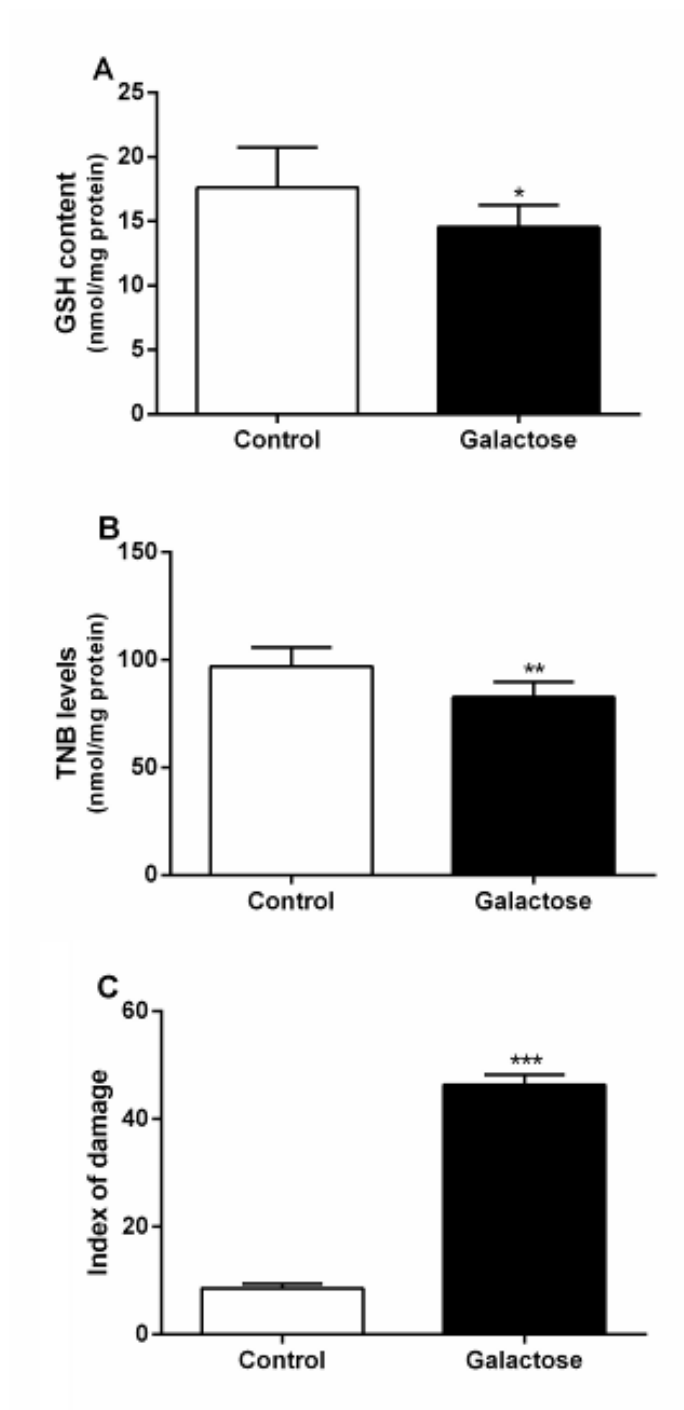


FIG. 7.



4. DISCUSSÃO

A GC é uma doença hereditária de origem recessiva, a sintomatologia desenvolvida nesta condição é múltipla, afetando vários tecidos (Fridovich-Keil and Walter, 2008). Com relação aos sintomas neurológicos, problemas cognitivos e motores são comumente desenvolvidos ao longo da vida dos pacientes, mesmo estes sendo submetidos a uma dieta restrita de GAL (Fridovich-Keil and Walter, 2008; Ridet et al., 2005). Porém, até o momento, as bases moleculares e celulares que desencadeiam a sintomatologia neurológica ainda não estão bem estabelecidas (Jumbo-Lucioni et al., 2012).

Além de ser acumulada no cérebro, a GAL pode ser produzida endogenamente e quando presente em altas concentrações pode desempenhar um importante papel neurotóxico na GC (Berry, 2011; Lai et al., 2009). Além disso, estudos com modelos experimentais de envelhecimento em ratos (Banji et al., 2013) ou camundongos (Cui et al., 2006), demonstraram que a administração crônica de altas concentrações de GAL é neurotóxica. Nesse contexto, no presente trabalho, padronizamos um modelo experimental, que é baseado na infusão de GAL diretamente no terceiro ventrículo de ratos Wistar através de injeção intracerebroventricular.

Nosso objetivo, primeiramente, foi investigar a influência da injeção intracerebroventricular de GAL sobre a memória aversiva e de reconhecimento de objetos. Além disso, investigamos a atividade, o imunoconteúdo e a expressão gênica da AChE em hipocampo e córtex cerebral dos animais submetidos a esse modelo. Os resultados encontrados mostraram que a administração

intracerebroventricular de GAL em ratos adultos prejudicou a fase de aquisição da memória aversiva e da memória de reconhecimento de objetos. Nossos resultados mostraram também um aumento na atividade da AChE em 1 h e 3 h, bem como uma diminuição na sua expressão gênica no hipocampo de ratos, 3 h após a administração intracerebroventricular de GAL.

O hipocampo e o córtex cerebral possuem uma importância fundamental nas diferentes etapas da formação da memória (Izquierdo and Medina, 1997; Squire, 1992). O sistema colinérgico desempenha diversas funções fisiológicas no SNC, tais como controle da inflamação (Pavlov et al., 2009; Scherer et al., 2014) e sobrevivência celular (Resende and Adhikari, 2009). O sistema colinérgico também desempenha um papel fundamental na formação, consolidação e uso da memória (Drever et al., 2011; Kukulja et al., 2009). Nesse contexto, a ação catalítica da AChE sobre a acetilcolina, desempenha uma importante função reguladora sobre a ação da acetilcolina em seus receptores (Pohanka, 2011).

Sabe-se que lesões e alterações químicas hipocampais podem provocar déficits de memória e aprendizado (Kim et al., 2002). Além disso, é bem estabelecido que o aumento da atividade da AChE está envolvido, pelo menos em parte, nos déficits cognitivos de muitas doenças neurodegenerativas (Mushtaq et al., 2014). Nesse contexto, o uso de inibidores da enzima AChE tem sido utilizados como importantes ferramentas no tratamento de doenças como Alzheimer, desde que essas substâncias são capazes de retardar a neurodegeneração, levando a um melhor prognóstico frente aos prejuízos de memória. Por isso, acredita-se que o sistema colinérgico desempenha um importante papel na sobrevivência celular (Akaike et al., 2010; Araujo et al., 2011; Resende and Adhikari, 2009).

Em resumo, os resultados encontrados no primeiro capítulo deste estudo sugerem que a injeção intracerebroventricular de GAL aumenta a atividade da AChE, o que pode estar contribuindo, pelo menos em parte, com os déficits cognitivos encontrados nas tarefas de memória realizadas. Esses achados estão de acordo com os resultados encontrados nos modelos de injeção crônica de GAL que mostram um aumento da atividade da AChE em diferentes estruturas cerebrais como hipocampo e córtex cerebral, desencadeando o déficit de memória (Kumar et al., 2010; Lu et al., 2010). Acreditamos que os resultados encontrados são relevantes, uma vez que, na primeira parte deste estudo mostramos que a administração intracerebroventricular de GAL, a uma concentração cerebral encontrada na patologia da GC, pode alterar a atividade e expressão gênica da AChE em hipocampo de ratos e assim promover déficit de memória, o qual comumente acomete os pacientes.

Na segunda parte do presente estudo, buscamos avaliar o efeito da injeção intracerebroventricular de GAL sobre a coordenação motora, bem como investigar alguns parâmetros bioquímicos e histológicos no cerebelo, devido ao papel central do cerebelo na coordenação do movimento (Apps and Garwicz, 2005). Nós encontramos um prejuízo na coordenação motora, bem como uma diminuição do número de células no espinocerebelo e cerebrocerebelo em ratos 24 h após a administração intracerebroventricular de GAL. Além disso, verificamos uma diminuição da imunofluorescência de NeuN e GFAP no espinocerebelo e de NeuN no cerebrocerebelo desses animais. A perda celular observada no cerebelo, especialmente a neuronal pode ter um grande impacto sobre a coordenação motora (He et al., 2013). A fim de melhor compreender os mecanismos pelo qual a

GAL levou a perda neuronal cerebelar, no presente estudo também investigamos parâmetros bioquímicos de morte celular. Vinte e quatro horas após a administração de GAL observamos: aumento do imunoconteúdo de caspase-3 ativa, diminuição dos níveis de BDNF, aumento da atividade da AChE e diminuição do imunoconteúdo desta enzima, diminuição dos níveis de GSH e sulfidrilas (dano proteico) e aumento dos níveis de dano ao DNA.

O cerebelo é uma estrutura central envolvida na coordenação motora. Recentes estudos têm mostrado que sua função se estende aos processos cognitivos (Apps and Garwicz, 2005; Manto et al., 2012; Ramnani, 2006). Assim, lesões cerebelares podem provocar déficits motores como: problemas de equilíbrio, postura e desordem e movimento dos membros (Manto et al., 2012). É importante salientar que na patologia da GC, atrofia cerebelares comumente são encontradas (Kaufman et al., 1995; Nelson et al., 1992). O primeiro grande estudo com pacientes de Waggoner e colaboradores (1990) mostrou que cerca de 20% dos pacientes são afetados por problemas motores, porém, este estudo foi baseado com a aplicação de questionários entre médicos. Atualmente, estudos sistemáticos mostram que o número de pacientes com problemas motores atinge em média 70% dos pacientes com GC (Potter et al., 2013; Rubio-Agusti et al., 2013).

O teste da trave é uma tarefa que permite avaliar a coordenação motora fina e equilíbrio em roedores. A disfunção motora pode ser induzida nestes animais farmacologicamente, geneticamente e/ou por injúria cerebral (Brooks and Dunnett, 2009; Luong et al., 2011; Wu and Gorantla, 2014). O resultado

comportamental encontrado no teste da trave mostra que a injeção intracerebroventricular de GAL provocou alterações motoras nos animais, o qual é compatível com o fenótipo desenvolvido por pacientes.

A seguir, nós avaliamos alguns parâmetros histológicos no cerebelo de ratos submetidos à injeção intracerebroventricular de GAL. A contagem manual de células em lâminas cerebelares marcadas com hematoxilina/eosina (H&E) mostrou que a GAL promoveu uma diminuição no número de células na camada molecular e granular da região espinocerebelar. Na região do cerebrocerebelo a GAL reduziu o número de células na camada molecular. Vale ressaltar que o espinocerebelo é responsável pelos movimentos do corpo e membros enquanto o cerebrocerebelo pelo planejamento dos movimentos (Kandel et al., 2013).

Também avaliamos o efeito da GAL sobre a população neuronal e astrogliar no espinocerebelo e cerebrocerebelo através da imunohistoquímica. Os resultados mostraram que a GAL provocou perda neuronal na camada granular do espinocerebelo e cerebrocerebelo, bem como perda de astrócitos no espinocerebelo. Os nossos resultados corroboram com estudos anteriores, que mostram um espalhamento da camada granular em um caso de autópsia Crome (1962) e suportam os achados de atrofia cerebelar comumente encontrada em pacientes (Kaufman et al., 1995; Nelson et al., 1992).

Circuitos neuronais são recrutados para realizar funções cerebrais, tais como função motora (Yuste, 2015). Os astrócitos são essenciais para a sobrevivência neuronal, principalmente por “alimentar” a população neuronal,

dessa forma o dano astrogliar pode desencadear patologias (Gundersen et al., 2015), uma vez que a morte de astrócitos é correlacionada com a perda neuronal (Schreiner et al., 2015). Assim, os resultados encontrados nas análises histológicas e imunohistoquímicas podem sugerir, que o problema motor encontrado é devido a morte celular em regiões cerebelares responsáveis pela coordenação do movimento. Os processos neurodegenerativos ocorrem ao longo de anos antes da morte do paciente, tornando difícil a elucidação dos mecanismos moleculares em casos de autópsia (Gorman, 2008).

O uso de modelos animais tem se mostrado uma importante ferramenta na elucidação dos mecanismos moleculares de diversas doenças (Delwing et al., 2003; Rodrigues et al., 2014; Wyse et al., 1995). Nesse contexto, nós prosseguimos o estudo avaliando alguns importantes marcadores bioquímicos para a função cerebral, afim de melhor compreender os eventos moleculares que desencadearam o prejuízo motor e a morte celular após a injeção intracerebroventricular de GAL.

A morte celular pode ser ativada pelo processo de necrose ou apoptose. A apoptose pode ser ativada por diferentes vias de sinalização, sendo estas intrínsecas ou extrínsecas, podendo ou não ser mediada por proteínas caspases. A apoptose mediada por caspase é a mais recorrente e a mais bem estudada. As caspases são sintetizadas como proteínas inativas e quando clivadas tornam-se ativas, acarretando na ativação sequencial de outras caspases (Hongmei, 2012; Nikolettópoulou et al., 2013). A caspase-3 é uma proteína central que desempenha um papel fundamental nas cascatas apoptóticas caspase dependente (Hongmei,

2012). Os resultados mostraram um aumento significativo no imunoconteúdo de caspase-3 ativa, sugerindo que a perda celular observada no cerebelo, após infusão intracerebroventricular de GAL, pode ser provocada por apoptose mediada por caspase.

O BDNF é uma importante neurotrofina envolvida na sobrevivência neuronal, participando ativamente de mecanismos de plasticidade sináptica, sendo que a produção desta neurotrofina é diretamente estimulada pela atividade neuronal (He et al., 2013; Zafra et al., 1992). As neurotrofinas protegem células neuronais da morte, o BDNF atua evitando a ativação de proteínas caspases (Kim and Zhao, 2005). Os níveis reduzidos de BDNF encontrados após a administração de GAL podem explicar a perda neuronal observada no espinocerebelo e cerebrocerebelo.

Além dos mecanismos de memória citados anteriormente, o sistema colinérgico desempenha outras funções no SNC tais como: sobrevivência celular, plasticidade sináptica entre outras (Resende and Adhikari, 2009). Assim, a enzima AChE tem sido muito estudada, devido seu papel finalizador da transmissão colinérgica (Pohanka, 2011). O aumento na atividade desta enzima implica na hipofunção colinérgica e está associada com doenças neurodegenerativas e déficit de memória (Ferreira et al., 2011; Mushtaq et al., 2014). Nesse contexto, tem sido mostrado que o uso de inibidores da AChE aumenta os níveis de BDNF e desacelera o processo neurodegenerativo pela promoção da sobrevivência neuronal (Bendix et al., 2014). O aumento da atividade da AChE, em nossos achados, pode estar correlacionado com a diminuição do BDNF encontrado. Nós

encontramos uma diminuição no imunoconteúdo da AChE, mas não sabemos ao certo as causas deste efeito, porém acreditamos que seja uma estratégia do tecido para reestabelecer a atividade enzimática.

A GSH é um tripeptídeo sintetizado endogenamente e a sua depleção está associada com apoptose e dano a biomoléculas (Aoyama and Nakaki, 2015; Circu and Aw, 2012; Halliwell, 2011). Nossos resultados mostraram que a injeção intracerebroventricular de GAL diminuiu os níveis de GSH em cerebelo de ratos, reforçando a hipótese que a GAL provoca apoptose, no modelo experimental utilizado. Estudos mostram que baixos níveis de GSH podem ser causados por desordens genéticas ou aumento na produção de radicais livres e parecem estar envolvidos com lesões cerebelares que desencadeiam déficit motor (Doss et al., 2015; Franco et al., 2006; Ristoff and Larsson, 2007). Além disso, a GAL aumentou o índice de dano ao DNA e a proteínas (diminuindo os níveis de sulfidrilas). Esses resultados, podem também explicar a morte celular observada nas análises histológicas.

Pacientes com GC geralmente desenvolvem problemas de coordenação motora tais como ataxia e tremor. Banji e colaboradores (2013), mostraram similarmente aos nossos resultados, que o tratamento crônico com GAL provocou déficit motor devido a alterações celulares e bioquímicas. Considerando que em nosso estudo foi utilizada uma concentração de GAL normalmente encontrada no SNC de pacientes, nossos achados contribuem para o entendimento da neurotoxicidade da GAL na fisiopatologia da GC.

Na segunda parte do presente estudo, mostramos que a GAL promove prejuízo na coordenação motora e morte celular em regiões cerebelares responsáveis pela sincronia do movimento. Além disso, foi mostrado que a perda celular é mediada, pelo menos em parte, por apoptose que pode ter sido fortemente influenciada pela diminuição dos níveis de BDNF e GSH, bem como pelo aumento da atividade da AChE e pelo dano à biomoléculas (DNA e proteínas). Tais resultados podem contribuir para um melhor entendimento da toxicidade da GAL sobre o cerebelo na GC.

Resumidamente, podemos sugerir que o modelo experimental desenvolvido foi capaz de reproduzir características comportamentais (memória e coordenação motora) normalmente encontradas comprometidas na doença da GC. Além disso, mostramos alterações em nível molecular e celular no hipocampo e cerebelo, provocadas pela administração intracerebroventricular de GAL. Esses resultados reforçam a importância da restrição dietética de GAL, também adicionam importantes informações à respeito do papel neurotóxico da GAL na patologia da GC.

5. CONCLUSÕES

- A administração intracerebroventricular de galactose em ratos:
 - ✓ Prejudicou a memória aversiva e de reconhecimento de objetos, além disso provocou no hipocampo:
 - Aumento na atividade da AChE;
 - Diminuição do mRNA da AChE.

 - ✓ Prejudicou a coordenação motora fina, além disso, alterou parâmetros histológicos e bioquímicos em cerebelo, tais como:
 - Diminuição no número de células, contadas por H&E;
 - Diminuição da imunomarcagem de astrócitos e neurônios;
 - Diminuição de GSH;
 - Aumento no dano de DNA e proteínas;
 - Aumento da caspase-3 ativa;
 - Diminuição do BDNF;
 - Aumento da atividade da AChE e diminuição de seu imunoconteúdo.

6. PERSPECTIVAS

- Investigar parâmetros de neuroinflamação em cerebelo e hipocampo, de ratos submetidos à injeção intracerebroventricular de GAL, tais como: TNF- α , IL-1 β e IL-6, bem como o imunocontéudo de IBA-1;
- Investigar o efeito da administração intracerebroventricular de GAL sobre parâmetros de metabolismo energético em cerebelo e hipocampo de ratos, tais como: massa mitocondrial e potencial de membrana mitocondrial, atividade das enzimas (complexo da piruvato desidrogenase, citrato sintase e dos complexos da cadeia respiratória, bem como os níveis de ATP);
- Investigar o efeito da administração intracerebroventricular de GAL sobre parâmetros de morte celular (anexina e PI) em cerebelo e hipocampo de ratos;
- Investigar o efeito da administração intracerebroventricular de galactose sobre a atividade, imunocontéudo e expressão gênica da Na⁺,K⁺-ATPase.

7. REFERÊNCIAS BIBLIOGRÁFICAS

- Akaike, A., Takada-Takatori, Y., Kume, T., Izumi, Y., 2010. Mechanisms of neuroprotective effects of nicotine and acetylcholinesterase inhibitors: role of alpha4 and alpha7 receptors in neuroprotection. *J. Mol. Neurosci.* 40, 211–216. doi:10.1007/s12031-009-9236-1
- Antunes, M., Biala, G., 2012. The novel object recognition memory: neurobiology, test procedure, and its modifications. *Cogn. Process.* 13, 93–110. doi:10.1007/s10339-011-0430-z
- Aoyama, K., Nakaki, T., 2015. Glutathione in Cellular Redox Homeostasis: Association with the Excitatory Amino Acid Carrier 1 (EAAC1). *Molecules* 20, 8742–8758. doi:10.3390/molecules20058742
- Applegarth, D.A., Toone, J.R., Lowry, R.B., 2000. Incidence of Inborn Errors of Metabolism in British Columbia, 1969-1996. *Pediatrics* 105, e10. doi:10.1542/peds.105.1.e10
- Apps, R., Garwicz, M., 2005. Anatomical and physiological foundations of cerebellar information processing. *Nat. Rev. Neurosci.* 6, 297–311. doi:10.1038/nrn1646
- Araujo, J.A., Greig, N.H., Ingram, D.K., Sandin, J., de Rivera, C., Milgram, N.W., 2011. Cholinesterase inhibitors improve both memory and complex learning in aged beagle dogs. *J. Alzheimers. Dis.* 26, 143–155. doi:10.3233/JAD-2011-110005
- Banji, D., Banji, O.J.F., Dasaroju, S., Kranthi, K.C.H., 2013. Curcumin and piperine

- abrogate lipid and protein oxidation induced by D-galactose in rat brain. *Brain Res.* 1515, 1–11. doi:10.1016/j.brainres.2013.03.023
- Bendix, I., Serdar, M., Herz, J., von Haefen, C., Nasser, F., Rohrer, B., Endesfelder, S., Felderhoff-Mueser, U., Spies, C.D., Sifringer, M., 2014. Inhibition of acetylcholinesterase modulates NMDA receptor antagonist mediated alterations in the developing brain. *Int. J. Mol. Sci.* 15, 3784–3798. doi:10.3390/ijms15033784
- Berry, G.T., 2011. Is prenatal myo-inositol deficiency a mechanism of CNS injury in galactosemia? *J. Inherit. Metab. Dis.* 34, 345–355. doi:10.1007/s10545-010-9260-x
- Berry, G.T., Nissim, I., Lin, Z., Mazur, A.T., Gibson, J.B., Segal, S., 1995. Endogenous synthesis of galactose in normal men and patients with hereditary galactosaemia. *Lancet* 346, 1073–1074.
- Bosch, A.M., 2011. Classic galactosemia: Dietary dilemmas. *J. Inherit. Metab. Dis.* 34, 257–260. doi:10.1007/s10545-010-9157-8
- Brooks, S.P., Dunnett, S.B., 2009. Tests to assess motor phenotype in mice: a user's guide. *Nat. Rev. Neurosci.* 10, 519–529. doi:10.1038/nrn2652
- Calderon, F.R.O., Phansalkar, A.R., Crockett, D.K., Miller, M., Mao, R., 2007. Mutation database for the galactose-1-phosphate uridylyltransferase (GALT) gene. *Hum. Mutat.* 28, 939–943. doi:10.1002/humu.20544
- Camelo Junior, J.S., Fernandes, M.I.M., Jorge, S.M., Maciel, L.M.Z., Santos, J.L.F., Camargo Junior, A.S., Passador, C.S., Camelo, S.H.H., 2011. Newborn screening for galactosemia: a health economics evaluation. *Cad. Saude Publica* 27, 666–676. doi:dx.doi.org/10.1590/S0102-

311X2011000400006

- Chen, Z., Simmons, M.S., Perry, R.T., Wiener, H.W., Harrell, L.E., Go, R.C.P., 2008. Genetic association of neurotrophic tyrosine kinase receptor type 2 (NTRK2) With Alzheimer's disease. *Am. J. Med. Genet. B. Neuropsychiatr. Genet.* 147, 363–369. doi:10.1002/ajmg.b.30607
- Circu, M.L., Aw, T.Y., 2012. Glutathione and modulation of cell apoptosis. *Biochim. Biophys. Acta* 1823, 1767–1777. doi:10.1016/j.bbamcr.2012.06.019
- Colettis, N.C., Snitcofsky, M., Kornisiuk, E.E., Gonzalez, E.N., Quillfeldt, J.A., Jerusalinsky, D. a, 2014. Amnesia of inhibitory avoidance by scopolamine is overcome by previous open-field exposure. *Learn. Mem.* 21, 634–645. doi:10.1101/lm.036210.114
- Coman, D.J., Murray, D.W., Byrne, J.C., Rudd, P.M., Bagaglia, P.M., Doran, P.D., Treacy, E.P., 2010. Galactosemia, a single gene disorder with epigenetic consequences. *Pediatr. Res.* 67, 286–292. doi:10.1203/PDR.0b013e3181cbd542
- Coss, K.P., Treacy, E.P., Cotter, E.J., Knerr, I., Murray, D.W., Shin, Y.S., Doran, P.P., 2014. Systemic gene dysregulation in classical Galactosaemia : Is there a central mechanism ? *Mol. Genet. Metab.* 113, 177–187. doi:10.1016/j.ymgme.2014.08.004
- Crome, L., 1962. A case of galactosaemia with the pathological and neuropathological findings. *Arch. Dis. Child.* 37, 415–421.
- Cui, X., Zuo, P., Zhang, Q., Li, X., Hu, Y., Long, J., Packer, L., Liu, J., 2006. Chronic systemic D-galactose exposure induces memory loss, neurodegeneration, and oxidative damage in mice: Protective effects of R- α -

- lipoic acid. *J. Neurosci. Res.* 84, 647–654. doi:10.1002/jnr.20899
- Dashty, M., 2013. A quick look at biochemistry: Carbohydrate metabolism. *Clin. Biochem.* 46, 1339–1352. doi:10.1016/j.clinbiochem.2013.04.027
- Deiana, S., Platt, B., Riedel, G., 2011. The cholinergic system and spatial learning. *Behav. Brain Res.* 221, 389–411. doi:10.1016/j.bbr.2010.11.036
- Delwing, D., Chiarani, F., Delwing, D., Bavaresco, C.S., Wannmacher, C.M.D., Wajner, M., Wyse, A.T.S., 2003. Proline reduces acetylcholinesterase activity in cerebral cortex of rats. *Metab. Brain Dis.* 18, 79–86.
- Devaraj, S., Hemarajata, P., Versalovic, J., 2013. The human gut microbiome and body metabolism: implications for obesity and diabetes. *Clin. Chem.* 59, 617–628. doi:10.1373/clinchem.2012.187617
- Dios, J.K.L., Capistrano-estrada, S., Chiong, M.A.D., 2009. Galactosemia in Three Filipino Patients - The Importance of Newborn Screening. *Acta Med. Philipp.* 1–3.
- Doss, S., Rinnenthal, J.L., Schmitz-Hübsch, T., Brandt, A.U., Papazoglou, S., Lux, S., Maul, S., Würfel, J., Endres, M., Klockgether, T., Minnerop, M., Paul, F., 2015. Cerebellar neurochemical alterations in spinocerebellar ataxia type 14 appear to include glutathione deficiency. *J. Neurol.* 262, 1927–1935. doi:10.1007/s00415-015-7788-2
- Drever, B.D., Riedel, G., Platt, B., 2011. The cholinergic system and hippocampal plasticity. *Behav. Brain Res.* 221, 505–514. doi:10.1016/j.bbr.2010.11.037
- Elmore, S., 2007. Apoptosis: a review of programmed cell death. *Toxicol. Pathol.* 35, 495–516. doi:10.1080/01926230701320337
- Elsas, L.J., Lai, K., 1998. The molecular biology of galactosemia. *Genet. Med.* 1,

40–48. doi:10.1097/00125817-199811000-00009

Elsa, S.H., Lucas, R.E., 2002. The Mousetrap: What We Can Learn When the Mouse Model Does Not Mimic the Human Disease. *ILAR J.* 43, 66–79. doi:10.1093/ilar.43.2.66

Ferreira, A.G.K., Scherer, E.B., da Cunha, M.J., Machado, F.R., da Cunha, A.A., Graeff, J.S., Netto, C.A., Wyse, A.T.S., 2011. Physical Exercise Reverses Cognitive Impairment in Rats Subjected to Experimental Hyperprolinemia. *Neurochem. Res.* 36, 2306–2315. doi:10.1007/s11064-011-0555-6

Franco, J.L., Teixeira, A., Meotti, F.C., Ribas, C.M., Stringari, J., Garcia Pomblum, S.C., Moro, Â.M., Bohrer, D., Bairros, A. V, Dafre, A.L., Santos, A.R.S., Farina, M., 2006. Cerebellar thiol status and motor deficit after lactational exposure to methylmercury. *Environ. Res.* 102, 22–28. doi:10.1016/j.envres.2006.02.003

Franco, R., Cidlowski, J.A., 2009. Apoptosis and glutathione: beyond an antioxidant. *Cell Death Differ.* 16, 1303–1314. doi:10.1038/cdd.2009.107

Fridovich-Keil, J.L., Gubbels, C.S., Spencer, J.B., Sanders, R.D., Land, J.A., Rubio-Gozalbo, E., 2011. Ovarian function in girls and women with GALT-deficiency galactosemia. *J. Inherit. Metab. Dis.* 34, 357–366. doi:10.1007/s10545-010-9221-4

Fridovich-Keil, J.L., Walter, J., 2008. Galactosemia, in: Valle, D., Beaudet, A., Vogelstein, B., Kinzler, K., Antonarakis, S., Ballabio, A. (Eds.), *The Online Metabolic & Molecular Bases of Inherited Disease*. McGrawHill, pp. 1–74.

Giovannini, M.G., Lana, D., Pepeu, G., 2015. The integrated role of ACh, ERK and mTOR in the mechanisms of hippocampal inhibitory avoidance memory. *Neurobiol. Learn. Mem.* 119, 18–33. doi:10.1016/j.nlm.2014.12.014

- Gitzelmann, R., 1995. Galactose-1-phosphate in the pathophysiology of galactosemia. *Eur. J. Pediatr.* 154, S45–S49. doi:10.1007/BF02143803
- Gorman, A.M., 2008. Neuronal cell death in neurodegenerative diseases: recurring themes around protein handling. *J. Cell. Mol. Med.* 12, 2263–2280. doi:10.1111/j.1582-4934.2008.00402.x
- Greber-Platzer, S., Guldberg, P., Scheibenreiter, S., Item, C., Schuller, E., Patel, N., Strobl, W., 1997. Molecular heterogeneity of classical and Duarte galactosemia: mutation analysis by denaturing gradient gel electrophoresis. *Hum. Mutat.* 10, 49–57. doi:10.1002/(SICI)1098-1004(1997)10:1<49::AID-HUMU7>3.0.CO;2-H
- Gundersen, V., Storm-Mathisen, J., Bergersen, L.H., 2015. Neuroglial Transmission. *Physiol. Rev.* 95, 695–726. doi:10.1152/physrev.00024.2014
- Guo, L., Li, B.X., Deng, M., Wen, F., Jiang, J.H., Tan, Y.Q., Song, Y.Z., Liu, Z.H., Zhang, C.H., Kobayashi, K., Wang, Z.N., 2011. Etiological analysis of neurodevelopmental disabilities: single-center eight-year clinical experience in south China. *J. Biomed. Biotechnol.* 2011. doi:10.1155/2011/318616
- Halliwell, B., 2012. Free radicals and antioxidants: Updating a personal view. *Nutr. Rev.* 70, 257–265. doi:10.1111/j.1753-4887.2012.00476.x
- Halliwell, B., 2011. Free radicals and antioxidants - Quo vadis? *Trends Pharmacol. Sci.* 32, 125–130. doi:10.1016/j.tips.2010.12.002
- Halliwell, B., 2006. Reactive species and antioxidants. Redox biology is a fundamental theme of aerobic life. *Plant Physiol.* 141, 312–322. doi:10.1104/pp.106.077073
- Hamamy, H., 2012. Consanguineous marriages: Preconception consultation in

- primary health care settings. *J. Community Genet.* 3, 185–192.
doi:10.1007/s12687-011-0072-y
- He, Y.Y., Zhang, X.Y., Yung, W.H., Zhu, J.N., Wang, J.J., 2013. Role of BDNF in central motor structures and motor diseases. *Mol. Neurobiol.* 48, 783–793.
doi:10.1007/s12035-013-8466-y
- Heimfarth, L., Loureiro, S.O., Dutra, M.F., Petenuzzo, L., de Lima, B.O., Fernandes, C.G., da Rocha, J.B.T., Pessoa-Pureur, R., 2013. Disrupted cytoskeletal homeostasis, astrogliosis and apoptotic cell death in the cerebellum of preweaning rats injected with diphenyl ditelluride. *Neurotoxicology* 34, 175–188. doi:10.1016/j.neuro.2012.10.015
- Henderson, H., Leisegang, F., Brown, R., Eley, B., 2002. The clinical and molecular spectrum of galactosemia in patients from the Cape Town region of South Africa. *BMC Pediatr.* 2, 7. doi:10.1186/1471-2431-2-7
- Hongmei, Z., 2012. Extrinsic and Intrinsic Apoptosis Signal Pathway Review, in: *Apoptosis and Medicine*. InTech, pp. 3–22. doi:10.5772/50129
- Isselbacher, K.J., Anderson, E.P., Kurahashi, K., Kalcar, H.M., 1956. Congenital galactosemia, a single enzymatic block in galactose metabolism. *Science* (80-). 123, 635–636.
- Izquierdo, I., Medina, J.H., 1997. Memory Formation: The Sequence of Biochemical Events in the Hippocampus and Its Connection to Activity in Other Brain Structures. *Neurobiol. Learn. Mem.* 68, 285–316.
doi:10.1006/nlme.1997.3799
- Izquierdo, L.A., Barros, D.M., Vianna, M.R.M., Coitinho, A., Silva, T.D., Choi, H., Moletta, B., Medina, J.H., Izquierdo, I., 2002. Molecular pharmacological

dissection of short- and long-term memory. *Cell. Mol. Neurobiol.* 22, 269–287.

doi:10.1023/A:1020715800956

Jumbo-Lucioni, P.P., Garber, K., Kiel, J., Baric, I., Berry, G.T., Bosch, A., Burlina, A., Chiesa, A., Pico, M.L.C., Estrada, S.C., Henderson, H., Leslie, N., Longo, N., Morris, A.A.M., Ramirez-Farias, C., Schweitzer-Krantz, S., Silao, C.L.T., Vela-Amieva, M., Waisbren, S., Fridovich-Keil, J.L., 2012. Diversity of approaches to classic galactosemia around the world: a comparison of diagnosis, intervention, and outcomes. *J. Inherit. Metab. Dis.* 35, 1037–1049. doi:10.1007/s10545-012-9477-y

Jumbo-Lucioni, P.P., Hopson, M.L., Hang, D., Liang, Y., Jones, D.P., Fridovich-Keil, J.L., 2013. Oxidative stress contributes to outcome severity in a *Drosophila melanogaster* model of classic galactosemia. *Dis. Model. Mech.* 6, 84–94. doi:10.1242/dmm.010207

Kandel, E.R., Schwartz, J.H., Jessell, T.M., Siegelbaum, S.A., Hudspetf, A.J., 2013. *Principles of Neural Science.*, 5th ed. McGraw-Hill, New York.

Kaufman, F.R., McBride-Chang, C., Manis, F.R., Wolff, J.A., Nelson, M.D., 1995. Cognitive functioning, neurologic status and brain imaging in classical galactosemia. *Eur. J. Pediatr.* 154, S2–5.

Kim, D.H., Zhao, X., 2005. BDNF Protects Neurons Following Injury by Modulation of Caspase Activity. *Neurocrit. Care* 3, 71–76. doi:10.1385/NCC:3:1:071

Kim, J.J., Diamond, D.M., Haven, N., Blvd, B.B.D., 2002. The stressed hippocampus, synaptic plasticity and lost memories. *Nat. Rev. Neurosci.* 3, 453–462. doi:10.1038/nrn849

Kukolja, J., Thiel, C.M., Fink, G.R., 2009. Cholinergic stimulation enhances neural

- activity associated with encoding but reduces neural activity associated with retrieval in humans. *J. Neurosci.* 29, 8119–8128. doi:10.1523/JNEUROSCI.0203-09.2009
- Kumar, A., Prakash, A., Dogra, S., 2010. Naringin alleviates cognitive impairment, mitochondrial dysfunction and oxidative stress induced by D-galactose in mice. *Food Chem. Toxicol.* 48, 626–632. doi:10.1016/j.fct.2009.11.043
- Kushner, R.F., Ryan, E.L., Sefton, J.M.I., Sanders, R.D., Lucioni, P.J., Moberg, K.H., Fridovich-Keil, J.L., 2010. A *Drosophila melanogaster* model of classic galactosemia. *Dis. Model. Mech.* 3, 618–627. doi:10.1242/dmm.005041
- Lai, K., Elsas, L.J., Wierenga, K.J., 2009. Galactose toxicity in animals. *IUBMB Life* 61, 1063–1074. doi:10.1002/iub.262
- Lai, K., Langley, S.D., Singh, R.H., Dembure, P.P., Hjelm, L.N., Elsas, L.J., 1996. A prevalent mutation for galactosemia among black Americans. *J. Pediatr.* 128, 89–95.
- Leslie, N.D., Immerman, E.B., Flach, J.E., Florez, M., Fridovich-Keil, J.L., Elsas, L.J., 1992. The human galactose-1-phosphate uridylyltransferase gene. *Genomics* 14, 474–480. doi:10.1016/S0888-7543(05)80244-7
- Leslie, N.D., Yager, K.L., McNamara, P.D., Segal, S., 1996. A mouse model of galactose-1-phosphate uridyl transferase deficiency. *Biochem. Mol. Med.* 59, 7–12. doi:10.1006/bmme.1996.0057
- Li, Y.N., Guo, Y., Xi, M.M., Yang, P., Zhou, X.Y., Yin, S., Hai, C.X., Li, J.G., Qin, X.J., 2014. Saponins from *Aralia taibaiensis* attenuate D-galactose-induced aging in rats by activating FOXO3a and Nrf2 pathways. *Oxid. Med. Cell. Longev.* 320513, 1–13. doi:10.1155/2014/320513

- Liu, J.X., Zhu, M.Y., Feng, C.Y., Ding, H.B., Zhan, Y., Zhao, Z., Ding, Y.M., 2015. Bamboo leaf extract improves spatial learning ability in a rat model with senile dementia. *J. Zhejiang Univ. Sci. B* 16, 593–601. doi:10.1631/jzus.B1400249
- Lu, B., 2003. Pro-Region of Neurotrophins. *Neuron* 39, 735–738. doi:10.1016/S0896-6273(03)00538-5
- Lu, J., Wu, D.M., Hu, B., Cheng, W., Zheng, Y.L., Zhang, Z.F., Ye, Q., Fan, S.H., Shan, Q., Wang, Y.J., 2010. Chronic administration of troxerutin protects mouse brain against D-galactose-induced impairment of cholinergic system. *Neurobiol. Learn. Mem.* 93, 157–164. doi:10.1016/j.nlm.2009.09.006
- Luong, T.N., Carlisle, H.J., Southwell, A., Patterson, P.H., 2011. Assessment of Motor Balance and Coordination in Mice using the Balance Beam. *J. Vis. Exp.* 2376. doi:10.3791/2376
- Mak, C.M., Lee, H.C.H., Chan, A.Y.W., Lam, C.-W., 2013. Inborn errors of metabolism and expanded newborn screening: review and update. *Crit. Rev. Clin. Lab. Sci.* 50, 142–162. doi:10.3109/10408363.2013.847896
- Manto, M., Bower, J.M., Conforto, A.B., Delgado-García, J.M., da Guarda, S.N.F., Gerwig, M., Habas, C., Hagura, N., Ivry, R.B., Mariën, P., Molinari, M., Naito, E., Nowak, D.A., Taib, N.O.B., Pelisson, D., Tesche, C.D., Tilikete, C., Timmann, D., 2012. Consensus Paper: Roles of the Cerebellum in Motor Control—The Diversity of Ideas on Cerebellar Involvement in Movement. *The Cerebellum* 11, 457–487. doi:10.1007/s12311-011-0331-9
- Mayatepek, E., Hoffmann, B., Meissner, T., 2010. Inborn errors of carbohydrate metabolism. *Best Pract. Res. Clin. Gastroenterol.* 24, 607–618. doi:10.1016/j.bpg.2010.07.012

- McGaugh, J.L., 2000. Memory--a century of consolidation. *Science* 287, 248–251.
- Mushtaq, G., Greig, N.H., Khan, J.A., Kamal, M.A., 2014. Status of acetylcholinesterase and butyrylcholinesterase in Alzheimer's disease and type 2 diabetes mellitus. *CNS Neurol. Disord. Drug Targets* 13, 1432–14329.
- Nasser, M., Javaheri, H., Fedorowicz, Z., Noorani, Z., 2009. Carnitine supplementation for inborn errors of metabolism, in: Nasser, M. (Ed.), *Cochrane Database of Systematic Reviews*. John Wiley & Sons, Ltd, Chichester, UK, p. CD006659. doi:10.1002/14651858.CD006659.pub2
- Nelson, M.D., Wolff, J.A., Cross, C.A., Donnell, G.N., Kaufman, F.R., 1992. Galactosemia: evaluation with MR imaging. *Radiology* 184, 255–261. doi:10.1148/radiology.184.1.1319076
- Nikoletopoulou, V., Markaki, M., Palikaras, K., Tavernarakis, N., 2013. Crosstalk between apoptosis, necrosis and autophagy. *Biochim. Biophys. Acta* 1833, 3448–3459. doi:10.1016/j.bbamcr.2013.06.001
- Ning, C., Reynolds, R., Chen, J., Yager, C., Berry, G.T., Leslie, N., Segal, S., 2001. Galactose metabolism in mice with galactose-1-phosphate uridylyltransferase deficiency: sucklings and 7-week-old animals fed a high-galactose diet. *Mol. Genet. Metab.* 72, 306–315. doi:10.1006/mgme.2001.3152
- Ning, C., Reynolds, R., Chen, J., Yager, C., Berry, G.T., McNamara, P.D., Leslie, N., Segal, S., 2000. Galactose metabolism by the mouse with galactose-1-phosphate uridylyltransferase deficiency. *Pediatr. Res.* 48, 211–217. doi:10.1203/00006450-200008000-00015
- Olsen, R.K.J., Cornelius, N., Gregersen, N., 2015. Redox signalling and mitochondrial stress responses; lessons from inborn errors of metabolism. *J.*

Inherit. Metab. Dis. 38, 703–719. doi:10.1007/s10545-015-9861-5

Padilla, C.D., Dans, L.F., Estrada, S.C., Tamondong, M.R., Laceste, J.J.O., Bernal, R.M.S., 2003. Cost-benefit analysis of newborn screening for galactosemia in the Philippines. *Southeast Asian J. Trop. Med. Public Health* 34 Suppl 3, 215–220.

Parameshwaran, K., Irwin, M.H., Steliou, K., Pinkert, C.A., 2010. D-Galactose Effectiveness in Modeling Aging and Therapeutic Antioxidant Treatment in Mice. *Rejuvenation Res.* 13, 729–735. doi:10.1089/rej.2010.1020

Pavlov, V.A., Parrish, W.R., Rosas-Ballina, M., Ochani, M., Puerta, M., Ochani, K., Chavan, S., Al-Abed, Y., Tracey, K.J., 2009. Brain acetylcholinesterase activity controls systemic cytokine levels through the cholinergic anti-inflammatory pathway. *Brain. Behav. Immun.* 23, 41–45. doi:10.1016/j.bbi.2008.06.011

Paxinos, G., Watson, C., 1986. *The Rat Brain in Stereotaxic Coordinates*. London.

Pohanka, M., 2011. Cholinesterases, a target of pharmacology and toxicology. *Biomed. Pap.* 155, 219–230. doi:10.5507/bp.2011.036

Potter, N.L., Nievergelt, Y., Shriberg, L.D., 2013. Motor and Speech Disorders in Classic Galactosemia. *JIMD Rep.* 11, 31–41. doi:10.1007/8904_2013_219

Quik, M., Zhang, D., Perez, X.A., Bordia, T., 2014. Role for the nicotinic cholinergic system in movement disorders; therapeutic implications. *Pharmacol. Ther.* 144, 50–59. doi:10.1016/j.pharmthera.2014.05.004

Ramnani, N., 2006. The primate cortico-cerebellar system: anatomy and function. *Nat. Rev. Neurosci.* 7, 511–522. doi:10.1038/nrn1953

Reichardt, J.K., Packman, S., Woo, S.L., 1991. Molecular characterization of two galactosemia mutations: correlation of mutations with highly conserved

- domains in galactose-1-phosphate uridyl transferase. *Am. J. Hum. Genet.* 49, 860–867.
- Resende, R.R., Adhikari, A., 2009. Cholinergic receptor pathways involved in apoptosis, cell proliferation and neuronal differentiation. *Cell Commun. Signal.* 7, 20. doi:10.1186/1478-811X-7-20
- Ridel, K.R., Leslie, N.D., Gilbert, D.L., 2005. An updated review of the long-term neurological effects of galactosemia. *Pediatr. Neurol.* 33, 153–161. doi:10.1016/j.pediatrneurol.2005.02.015
- Ristoff, E., Larsson, A., 2007. Inborn errors in the metabolism of glutathione. *Orphanet J. Rare Dis.* 2, 16. doi:10.1186/1750-1172-2-16
- Rodrigues, A.F., Roecker, R., Junges, G.M., de Lima, D.D., da Cruz, J.G.P., Wyse, A.T.S., Dal Magro, D.D., 2014. Hypoxanthine induces oxidative stress in kidney of rats: protective effect of vitamins E plus C and allopurinol. *Cell Biochem. Funct.* 32, 387–394. doi:10.1002/cbf.3029
- Rubio-Agusti, I., Carecchio, M., Bhatia, K.P., Kojovic, M., Parees, I., Chandrashekar, H.S., Footitt, E.J., Burke, D., Edwards, M.J., Lachmann, R.H.L., Murphy, E., 2013. Movement Disorders in Adult Patients With Classical Galactosemia. *Mov. Disord.* 28, 804–810. doi:10.1002/mds.25348
- Ryan, E.L., DuBoff, B., Feany, M.B., Fridovich-Keil, J.L., 2012. Mediators of a long-term movement abnormality in a *Drosophila melanogaster* model of classic galactosemia. *Dis. Model. Mech.* 5, 796–803. doi:10.1242/dmm.009050
- Scherer, E.B.S., Loureiro, S.O., Vuaden, F.C., da Cunha, A.A., Schmitz, F., Kolling, J., Savio, L.E.B., Bogo, M.R., Bonan, C.D., Netto, C.A., Wyse, A.T.S., 2014. Mild Hyperhomocysteinemia Increases Brain Acetylcholinesterase and

Proinflammatory Cytokine Levels in Different Tissues. *Mol. Neurobiol.* 50, 589–596. doi:10.1007/s12035-014-8660-6

Schreiner, B., Romanelli, E., Liberski, P., Ingold-Heppner, B., Sobottka-Brillout, B., Hartwig, T., Chandrasekar, V., Johannssen, H., Zeilhofer, H.U., Aguzzi, A., Heppner, F., Kerschensteiner, M., Becher, B., 2015. Astrocyte Depletion Impairs Redox Homeostasis and Triggers Neuronal Loss in the Adult CNS. *Cell Rep.* 12, 1377–1384. doi:10.1016/j.celrep.2015.07.051

Schulpis, K.H., Michelakakis, H., Tsakiris, T., Tsakiris, S., 2005. The effect of diet on total antioxidant status, erythrocyte membrane Na⁺,K⁺-ATPase and Mg²⁺-ATPase activities in patients with classical galactosaemia. *Clin. Nutr.* 24, 151–157. doi:10.1016/j.clnu.2004.09.001

Schulpis, K.H., Papassotiriou, I., Tsakiris, S., 2006. 8-hydroxy-2-desoxyguanosine serum concentrations as a marker of DNA damage in patients with classical galactosaemia. *Acta Paediatr.* 95, 164–169. doi:10.1080/08035250500297810

Schwartz, P.M., Borghesani, P.R., Levy, R.L., Pomeroy, S.L., Segal, R.A., 1997. Abnormal cerebellar development and foliation in BDNF^{-/-} mice reveals a role for neurotrophins in CNS patterning. *Neuron* 19, 269–281.

Scriver, C., 2008. Garrod's Croonian Lectures (1908) and the charter "Inborn Errors of Metabolism": albinism, alkaptonuria, cystinuria, and pentosuria at age 100 in 2008. *J. Inherit. Metab. Dis.* 31, 580–98. doi:10.1007/s10545-008-0984-9

Scriver, C., 2001. *The metabolic & molecular bases of inherited disease*. McGraw-Hill.

Shih, L.Y., Suslak, L., Rosin, I., Searle, B.M., Desposito, F., 1984. Gene dosage

- studies supporting localization of the structural gene for galactose-1-phosphate uridyl transferase (GALT) to band p13 of chromosome 9. *Am. J. Med. Genet.* 19, 539–543. doi:10.1002/ajmg.1320190316
- Slepek, T., Tang, M., Addo, F., Lai, K., 2005. Intracellular galactose-1-phosphate accumulation leads to environmental stress response in yeast model. *Mol. Genet. Metab.* 86, 360–371. doi:10.1016/j.ymgme.2005.08.002
- Slepek, T.I., Tang, M., Slepek, V.Z., Lai, K., 2007. Involvement of endoplasmic reticulum stress in a novel Classic Galactosemia model. *Mol. Genet. Metab.* 92, 78–87. doi:10.1016/j.ymgme.2007.06.005
- Squire, L.R., 1992. Memory and the hippocampus: a synthesis from findings with rats, monkeys, and humans. *Psychol. Rev.* 99, 195–231. doi:dx.doi.org/10.1037/0033-295X.99.2.195
- Tsakiris, S., Carageorgiou, H., Schulpis, K.H., 2005. The protective effect of L-cysteine and glutathione on the adult and aged rat brain (Na⁺,K⁺)-ATPase and Mg²⁺-ATPase activities in galactosemia in vitro. *Metab. Brain Dis.* 20, 87–95. doi:10.1007/s11011-005-2480-3
- Tsakiris, S., Marinou, K., Schulpis, K.H., 2002. The in vitro Effects of Galactose and its Derivatives on Rat Brain Mg²⁺-ATPase Activity. *Pharmacol. Toxicol.* 91, 254–257. doi:10.1034/j.1600-0773.2002.910506.x
- Valko, M., Leibfritz, D., Moncol, J., Cronin, M.T.D., Mazur, M., Telser, J., 2007. Free radicals and antioxidants in normal physiological functions and human disease. *Int. J. Biochem. Cell Biol.* 39, 44–84. doi:10.1016/j.biocel.2006.07.001
- Waggoner, D.D., Buist, N.R.M., Donnell, G.N., 1990. Long-term prognosis in

- galactosaemia: Results of a survey of 350 cases. *J. Inherit. Metab. Dis.* 13, 802–818. doi:10.1007/BF01800204
- Walter, J.H., Fridovich-Keil, J.L., 2014. Galactosemia, in: Beaudet, A.L., Vogelstein, B., Kinzler, K.W., Antonarakis, S.E., Ballabio, A., Gibson, K.M., Mitchell, G. (Eds.), *The Online Metabolic and Molecular Bases of Inherited Disease*. The McGraw-Hill Companies, Inc., New York, NY.
- Wu, L., Gorantla, S., 2014. *Current Laboratory Methods in Neuroscience Research* 115–128. doi:10.1007/978-1-4614-8794-4
- Wyse, A.T., Sarkis, J.J., Cunha-Filho, J.S., Teixeira, M. V, Schetinger, M.R., Wajner, M., Wannmacher, C.M., 1995. ATP diphosphohydrolase activity in synaptosomes from cerebral cortex of rats subjected to chemically induced phenylketonuria. *Braz. J. Med. Biol. Res.* 28, 643–649.
- Yuste, R., 2015. From the neuron doctrine to neural networks. *Nat. Rev. Neurosci.* 16, 487–497. doi:10.1038/nrn3962
- Zafra, F., Lindholm, D., Castrén, E., Hartikka, J., Thoenen, H., 1992. Regulation of brain-derived neurotrophic factor and nerve growth factor mRNA in primary cultures of hippocampal neurons and astrocytes. *J. Neurosci.* 12, 4793–4799.
- Zuccato, C., Cattaneo, E., 2009. Brain-derived neurotrophic factor in neurodegenerative diseases. *Nat. Rev. Neurol.* 5, 311–22. doi:10.1038/nrneurol.2009.54